Adrenergic and ischaemic challenge on the activities of lipid metabolising enzymes in the perfused rat heart.

Heathers, Guy Phillip

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Submitted by Guy Phillip Heathers

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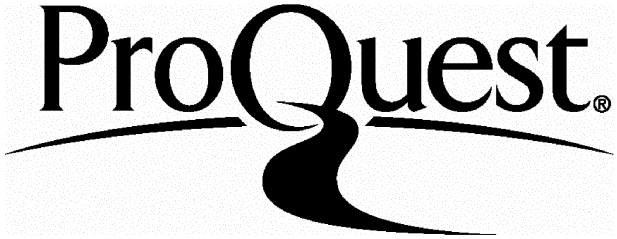
of the University of Bath.

1985.



Dedicated to Aunty Mum.

Acknowledgements

I am greatly indepted to Dr. R.V. Brunt for advice, criticism and encouragement throughout the project. I would also like to thank Dr. B. Woodward for his advice and help and Dr. P. England for providing the catalytic subunit of cAMP-dependent protein kinase.

Lastly, I would like to express my gratitude to my parents for their continued support and encouragement throughout the years.

CONTENTS

Summary

This investigation was undertaken to study the effects of adrenergic and ischaemic challenge on the activity of lipid metabolising enzymes in the perfused rat heart.

Perfusion with adrenaline or the beta agonist isoprenaline produced an increase in triglyceride lipase (TGL) activity and a fall in glycerol 3-phosphate acyltransferase (GPAT) activity. No change was seen in carnitine palmitoyl transferase (CPT) activity. These changes could be imitated by incubation of heart homogenates with cAMP-dependent protein kinase. The non-selective alpha agonist phenylephrine, and the alpha$_2$ agonist clonidine produced the opposite affect, a fall in TGL activity and a rise in GPAT activity. Methoxamine, an alpha$_1$ agonist, had no affect on TGL activity but reduced GPAT activity. Changes in GPAT activity were localized mainly in the microsomal fraction. The changes in TGL and GPAT activity are consistant with both enzymes being regulated via a cyclic AMP-dependent protein kinase system and via alpha adrenergic mechanisms.

Ischaemia was produced by occlusion of the left descending coronary artery for 10 minutes. Compared to activities measured in tissue from normally perfused hearts, GPAT activity measured in tissue from the ischaemic area was considerably reduced while TGL activity in the ischaemic area was markedly increased. No change was seen in GPAT or TGL activity measured in tissue from the non-ischaemic area

or in CPT activity measured in both areas. The changes in activities produced by ischaemia were prevented by pre-perfusion with the cardio-selective beta antagonist Atenolol.

Reperfusion of the ischaemic area resulted in TGL activity returning to the value measured in tissue from normally perfused hearts. However, GPAT activity, after 1 minute of reperfusion, fell to a value lower than after 10 minutes ischaemia. This reperfusion-induced fall in GPAT activity was prevented by pre-perfusion with the $alpha_1$ antagonist Doxasozin. Pre-perfusion of the $alpha_2$ antagonist Yohimbine resulted in a prolongation of the increased TGL activity in the ischaemic area during reperfusion. All changes in enzyme activities were prevented when endogenous noradrenaline stores were depleted by injection of 6 OH-dopamine 24 hours before hearts were removed.

These changes in enzyme activities show that during ischaemia there is an increased beta-adrenergic drive. On reperfusion the beta-adrenergic drive is removed but an $alpha_1$ adrenergic drive becomes apparent.

INTRODUCTION

Section 1.1.

The Mammalian Heart

i. Gross Structure, Blood Supply and Innervation.

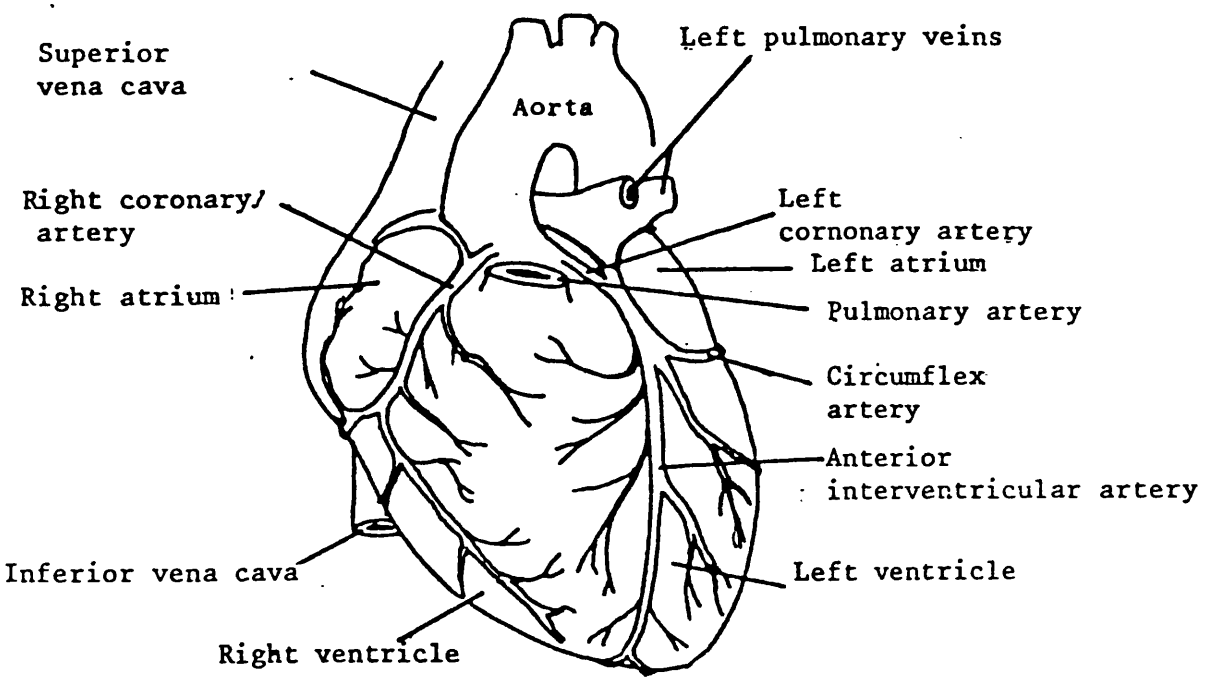
The heart is a muscular organ located in the thorax. It is composed of four chambers; two upper chambers, the left and right atria and two lower chambers, the left and right ventricles. The right atrium recieves blood from the rest of the body. The blood is delivered to the lungs by contraction of the right ventricle. Oxygenated blood returns from the lungs to the left atrium. It is pumped around the rest of the body by the left ventricle. The atria, which recieve blood, are delicate compared to the ventricles which drive blood into the circulations. The left ventricle is more powerful than the right ventricle (with thicker walls) to enable it to develop the high pressure necessary to drive blood into the aorta and around the body. Of the total heart muscle about two thirds is represented by the left ventricular muscle. Fig 1.1 shows the external anatomy and the different compartments of the mammalian heart.

Heart tissue, as any other highly active tissue, requires an extensive blood supply to deliver substrates and remove products. The circulation begins with coronary arteries which branch from the aorta just after the position of the aortic valve. The highly oxygenated blood perfuses the outer surface of the myocardium, the subepicardial

Fig.1.1

The external anatomy of the heart

The anterior surface



region, first. From here the vessels branch into the mid and subendocardial regions. Coronary flow is mainly restricted to the period between contractions (the diastolic period) since, during contractions (the systolic period) the increase in myocardial wall tension causes extensive occlusion of the coronary vessels. The occlusion is most extensive and of longest duration in the subendocardial region.

The venous system of the myocardium conveys most of the blood to the right atrium via the coronary sinus. A small but significant drainage of blood occurs directly into the chambers through the thebesian and other small veins.

Section 1.2.

Histology and Physiology of Cardiac Muscle

i. The Cardiac Muscle

The muscular walls of the ventricles consist of three layers (fig 1.2). The inner layer called the endocardium, a middle layer called the myocardium and an outer layer called the epicardium. The layer of tissue surrounding the epicardium is called the pericardium.

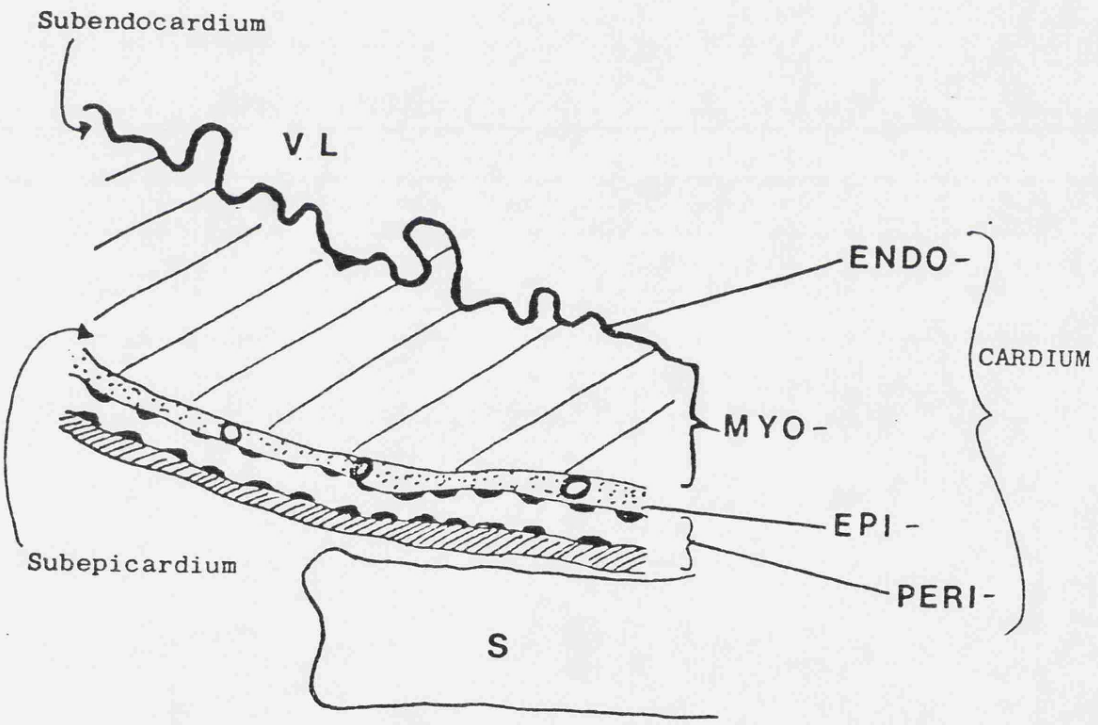
ii. The Ultrastructure of the Myocardium.

The ultrastructure of the myocardium is shown in fig 1.3. The surface membrane of the myocardial cell, the sarcolemma, consists of a basement membrane and a plasma

Figure 1·2

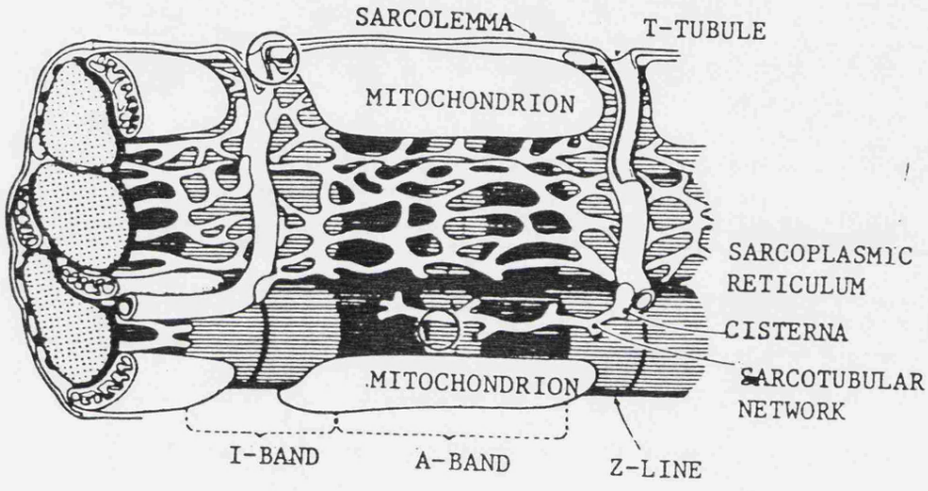
CROSS-SECTION OF HEART WALL

VL = ventricular lumen
S = Sternum



Adapted from Muir, 1971.

Figure 1·3



From Katz (1977).

membrane. The basement membrane is composed of glycoproteins (Scott, 1968 and Howse et al, 1970) and has the capacity to bind Ca^{2+} ions which are important for contraction (Dhalla et al, 1977). The enzymes involved in ion pumping are located in the plasma membrane in which depolarisation, excitation and contraction are initiated.

Invaginations of the sarcolemma form the sarcoplasmic reticulum. It consists of the sarcolemmal cisternae and the sarcotubular network. The cisternae are located under the sarcolemma and alongside the transverse tubular system (T-system). The T-system is open to the extracellular space and runs alongside the Z-band in cardiac muscle.

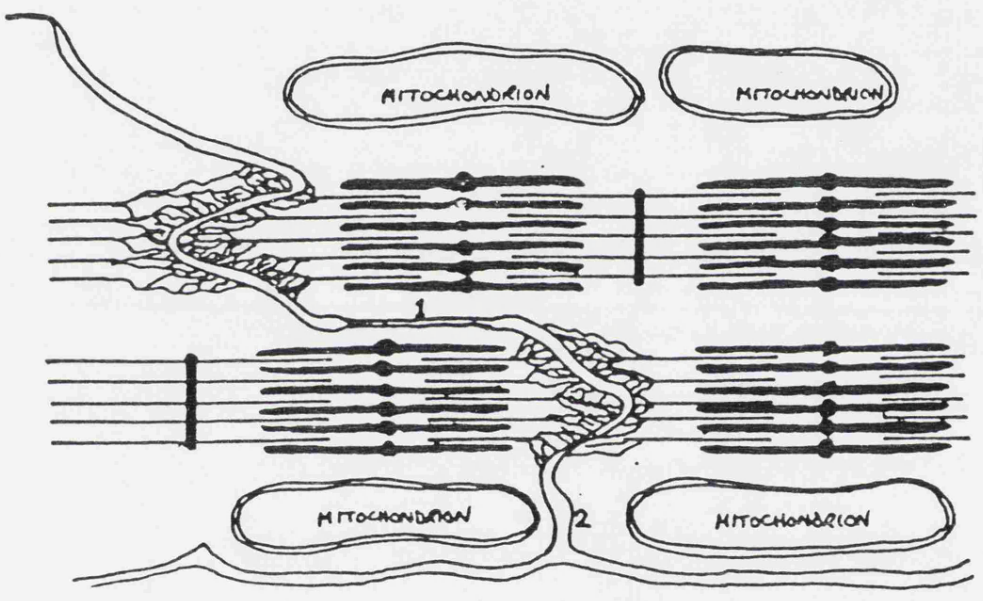
The sarcoplasmic reticulum has two important functions (Dhalla et al, 1977,78): the release of Ca^{2+} ions to the contractile elements following depolarisation of the sarcolemma and the T-system, and the reabsorbtion of Ca^{2+} ions from the contractile elements in order to bring about relaxation.

The basic contractile unit in cardiac muscle is called the sarcomere. Sarcomeres are joined end to end to form myofibrils, the structure of which is shown in fig 1.4. Myofibrils have a dark band called the A-band which corresponds to myosin and the I-band which corresponds to actin. During contraction, the actin molecules slide along the myosin molecules.

The cardiac cell contains large numbers of mitochondria, illustrating the highly oxidative nature of myocardial metabolism. Glycogen granules and lipid droplets are also seen and represent internal stores of energy (Stein

Figure 1·4

Diagram of muscle fibre as seen by electron microscopy showing an intercalated disc



1 nexus

2 intercellular gap

From Bloor (1978).

and Stein, 1963).

iii.Actin-Myosin Interaction

Bound to the actin molecules are molecules of tropomyosin and troponin. The actin-myosin interactions are modulated by Ca^{2+}, troponin and tropomyosin. There are 3 troponin proteins, troponin-I (TnI), troponin-C (TnC) and troponin-T (TnT). When Ca^{2+} binds to TnC, interaction of actin and myosin is facilitated. TnI inhibits the actin-myosin interaction, the inhibition is reversed when TnC binds Ca^{2+} (Katz,1977). Fig 1.5 shows that the cycle of contraction and relaxation, involving actin and myosin, requires energy. This is provided by the breakdown of ATP through the myosin-bound ATPase. Contraction is believed to occur when the intracellular Ca^{2+} rises and uptake of Ca^{2+} by TnC occurs. Relaxation occurs by the active uptake of Ca^{2+} ions by the sarcoplasmic reticulum leading to a decrease in the intracellular Ca^{2+} concentration.

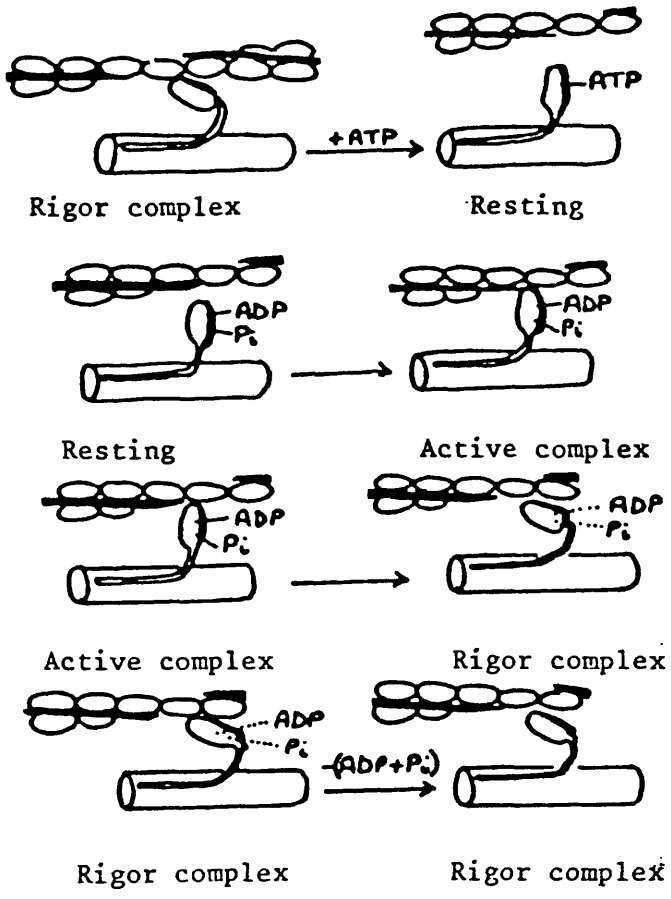
iv. Electrical Properties

In addition to the classical muscular function of contraction, the heart exhibits other properties. These include automaticity (the ability to contract independently of external stimulation) and conductivity (electrical impulses passed from one cardiac cell to another without intervention of nervous tissue).

Unlike skeletal muscle, the myocardium acts as a syncytium allowing the coordination of contraction. This is

Figure 1·5

ATP interactions with the contractile elements



From Katz (1977).

vital to the pumping action of the heart. Electrical impulses originate on a group of specialised cells, called the sinus node, located on the surface of the right atrium near its junction with the superior vena cava. The node depolarises and repolarises at a constant rate which (usually) determines the heart rate. The sinus node provides the usual dominant heart rhythm but automaticity is also exhibited by the ventricular muscle itself in the absence of sinus rhythm.

The impulse from the sinus node spreads across the surface of the atria, causing them to contract and then passes rapidly to the apex of the ventricles via the atrioventricular node and the Purkinje fibres. These are muscle cells which, through specialisation, have lost the ability to contract but instead conduct electrical impulses back up through the bulk of the ventricular muscle mass, initiating the rising wave of ventricular contraction.

The electrical wave passes from one muscle cell to the next in a way analagous to nervous transmission along axons. The cells communicate at sites called intercalated discs.

Section 1.3.

The Metabolism of the Heart

Several reviews have described the metabolism of the heart under normal, ischaemic and hypoxic conditions in considerable detail. In partcular, the articles by Bing (1965), Wollenberger and Krause (1968), Opie (1968,69), Kubler and Spieckerman (1970), Neely, Rovetto and Oram (1972a,b), Neely and Morgan (1974), Gibbs (1978) and two articles on ischaemia by Braunwald (1976) and Wildenthal et al (1976) cover the area.

i. Substrates Utilized

The heart is a constantly-acting muscular tissue and is therefore a centre of intense metabolic activity. The substrates required for combustion, to provide the energy needed for mechanical and electrical activity, are supplied largely by the blood. The heart normally depends largely upon exogenous substrates. Under normal conditions the glycogen and lipid stored within the myocardium (endogenous substrates) are not extensively utilised. However, under certain conditions the balance between utilization of endogenous and exogenous fuels can shift markedly. In particular, the utilization of endogenous lipid may become significant.

ii. Exogenous Substrates

The heart utilizes both glucose and fatty acids as sources of energy. However, fatty acids are the preferred substrates (Evans, 1934; Cruickshank et al, 1936,41 and Opie, 1968). In the human heart 75% of the oxygen consumption can be accounted for by oxidation of plasma free fatty acids (FFA) (Most et al, 1969 and Lassers et al, 1971,72). Under most conditions fatty acids are oxidised in preference to carbohydrate and their oxidation normally accounts for 60-70% of oxidative metabolism (Neely et al, 1972a,b). When FFA are available, they suppress the utilisation of glucose, probably by inhibition of steps either in the glycolytic pathway (Shipp et al, 1961 and Opie, 1968) or at pyruvate dehydrogenase (PDH), as acetyl CoA units formed from the oxidation of FFA would decrease PDH activity and thus glucose oxidation (Randle et al, 1970; Denton et al, 1972 and Martin et al, 1972).

Mono-unsaturated fatty acids are claimed to be utilised more rapidly than saturated or di unsaturated fatty acids (Evans, 1964 and Willebrands, 1964). However, these studies have been challenged by Stein and Stein (1963) since the A-V difference in FFA composition of the blood may be altered by exchange reactions catalysed by lipoprotein lipase (LPL, see below), with no necessity for net FFA uptake. Indeed using isolated perfused rat hearts, no selectivity in FFA uptake could be observed (Stein and Stein, 1963).

Lipid is also supplied to the heart as triglyceride-fatty acids (TGFA). TGFA are present in the blood mainly in the form of chylomicra and very low density

lipoproteins (VLDL). Both these forms are complexes containing approximately similar amounts of triglyceride (60%), phospholipid (15%), cholesterol (10%), cholesterol esters (5%) and protein (10%) (Fredickson, 1974). The chylomicra derive their lipid directly from dietary lipid in the intestinal mucosa. VLDL's are synthesised in the liver and are discharged into the blood.

The utilization of TGFA proceeds by hydrolysis at the cell surface to release FFA which then enter the cell (Crass et al, 1965). This hydrolysis is catalysed by LPL.

In addition to the two major substrates, the heart can also utilize lactate, pyruvate, ketones and amino acids. These under most conditions may not be major substrates (Olson, 1962).

iii. Endogenous Substrates

Although exogenous substrates comprise the major source of energy for the heart, the heart can also utilize a variety of endogenous substrates as energy sources. The utilization of endogenous fuel was demonstrated by substrate-free perfusion of the isolated rat heart (Shipp et al, 1964 and Olson and Hoeschen, 1967). Glycogen was depleted within 5 minutes but further contraction was maintained by endogenous lipid for up to 45 minutes. This was confirmed by the production of $^{14}CO_2$ from prelabelled lipids (Shipp et al, 1964).

The role of phospholipid as an endogenous energy source is uncertain (Crass et al, 1969,71).

The rate of endogenous triglyceride utilization can

be increased by an increased work-load performed by the heart or catecholamine stimulation. The latter appears to be mediated by a higher level of cyclic AMP and activation of an intracellular lipase by protein kinase (Vavrinkova and Mosinger, 1974). This is in contrast to the increased lipolysis induced by increased cardiac work, which is associated with a lower tissue level of fatty acid (Oram et al, 1973). Exogenous glucose has little effect on the degree of endogenous triglyceride utilization, but exogenous FFA appear to inhibit nett endogenous lipolysis (Crass et al, 1975 and Crass and Sterrett, 1975). Elevated levels of endogenous fatty acids or long-chain acyl CoA level have also been shown to inhibit lipolysis (Neely et al, 1972b).

Section 1.4

Intracellular Metabolism of the Heart.

Major Pathways

The highly aerobic nature of myocardial metabolism exhibited under all but extreme conditions is demonstrated by the large number of mitochondria which make up about 30% of the cardiac cell volume (Sobel, 1974). Other indices of the aerobic nature of the cardiac metabolism are the capacity of the heart to utilise lactic acid (Opie, 1968) and the high activity of oxidative enzymes, particularly cytochromes (Penpargkul and Scheuer, 1970).

The major pathways for generation of energy in the myocardium are the normal patways of carbohydrate and lipid utilization (Mahler and Cordes, 1966). These are illustrated in figs 1.6 and 1.7, and are briefly outlined here.

i. Glucose Metabolism

Insulin facilitates the transport of glucose across the cell membrane into the cytoplasm of the myocardial cell. Once inside, the glucose is rapidly phosphorylated by hexokinase to glucose-6-phosphate (Morgan et al, 1961; Morgan and Parmeggiani, 1964; Opie et al, 1973 and Neely and Morgan, 1974).

Glucose-6-phosphate may be polymerised and stored as glycogen (glycogenesis). Subsequently, glycogen breakdown

Legend for Figure 1·6

1. Glucose kinase, hexokinase
2. Phosphoglucose isomerase
3. Phosphoglucomutase
4. Phosphofructokinase
5. Aldolase
6. Triosephosphate isomerase
7. Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)
8. Phosphoglycerate kinase
9. Phosphoglyceratemutase
10. Enolase
11. Pyruvate kinase
12. Lactic dehydrogenase
13. Pyruvate dehydrogenase

Figure 1·6

Glycolytic pathway

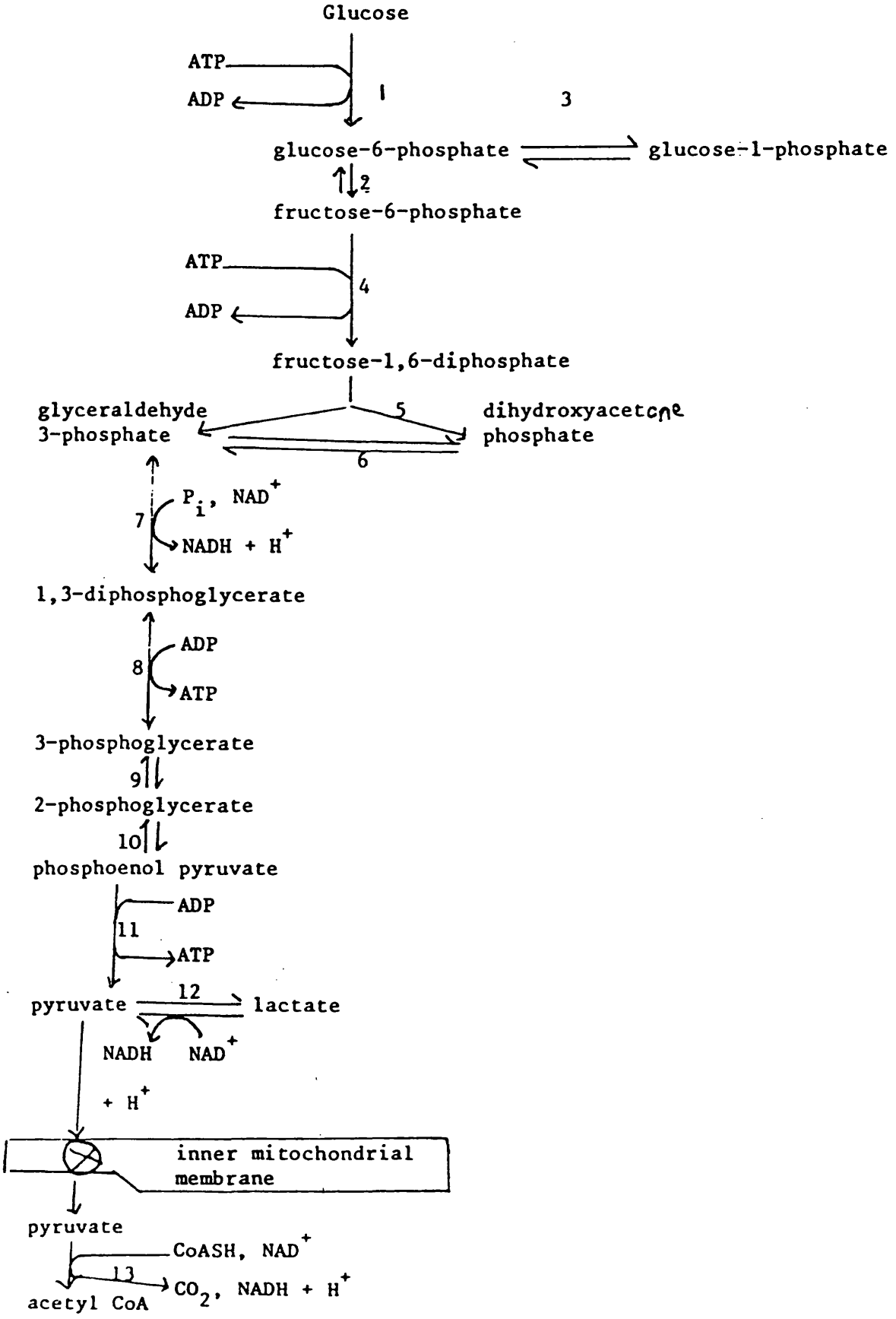
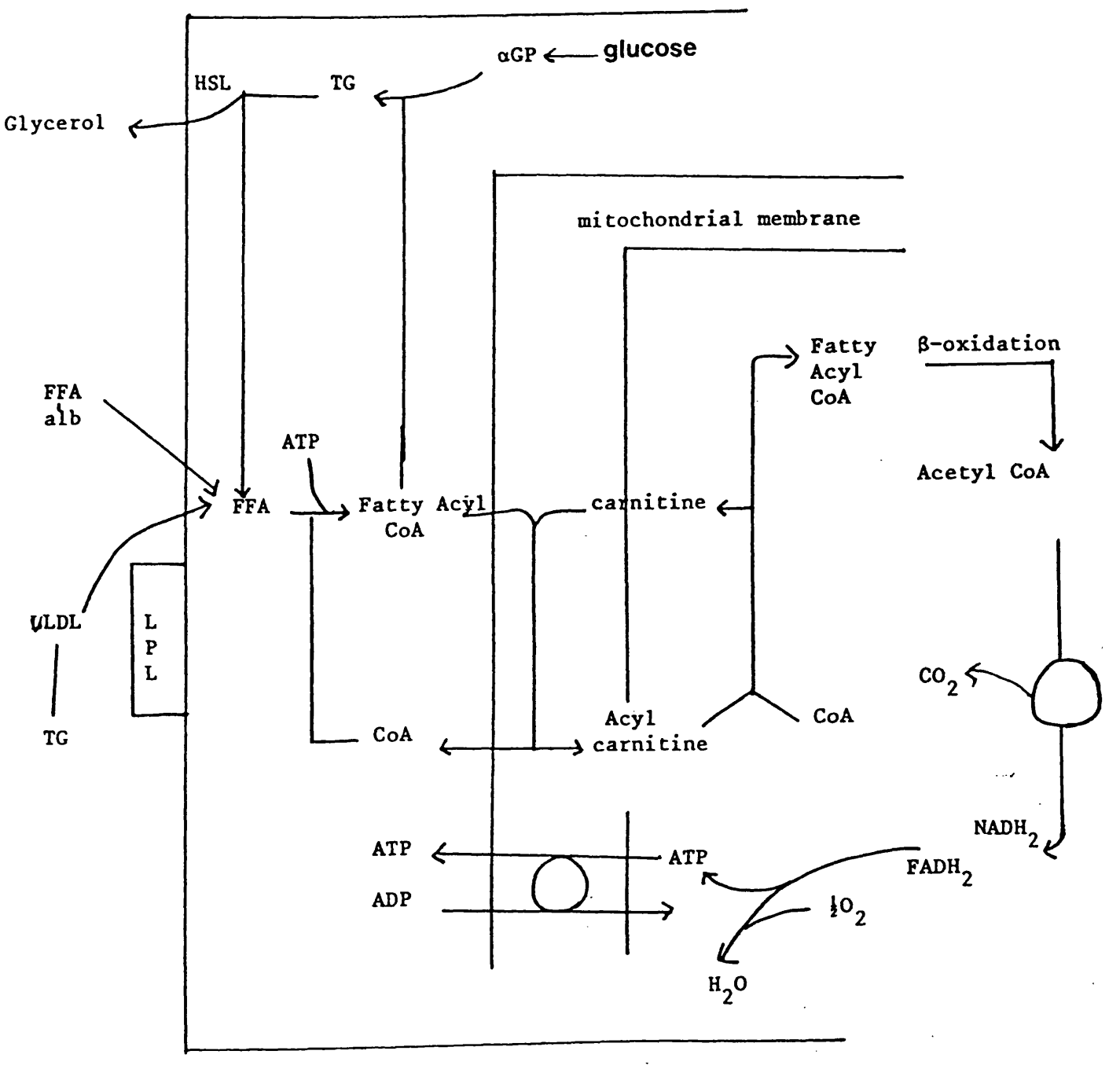


Figure 1·7

Lipid metabolism in cardiac muscle



From Severson (1979) Can. J. Physiol. Pharmacol.

occurs in an energy-yielding process called glycolysis (Opie et al, 1963 and Opie, 1968). The Embden-Meyerhof pathway of glycolysis is the major pathway of degradation of glucose-6-phosphate to yield energy. The end products are, under aerobic conditions, pyruvate or, under reduced oxygen supply, lactate.

The activity of phosphofructokinase (PFK) provides an important control point in the regulation of glycolysis in the heart. ATP and citrate reduce the activity of this enzyme and thus exert a restriction on the rate of glycolysis when these compounds are of a sufficiently high concentration i.e. during energy surplus (Mansour, 1963).

ii. Pyruvate Dehydrogenase

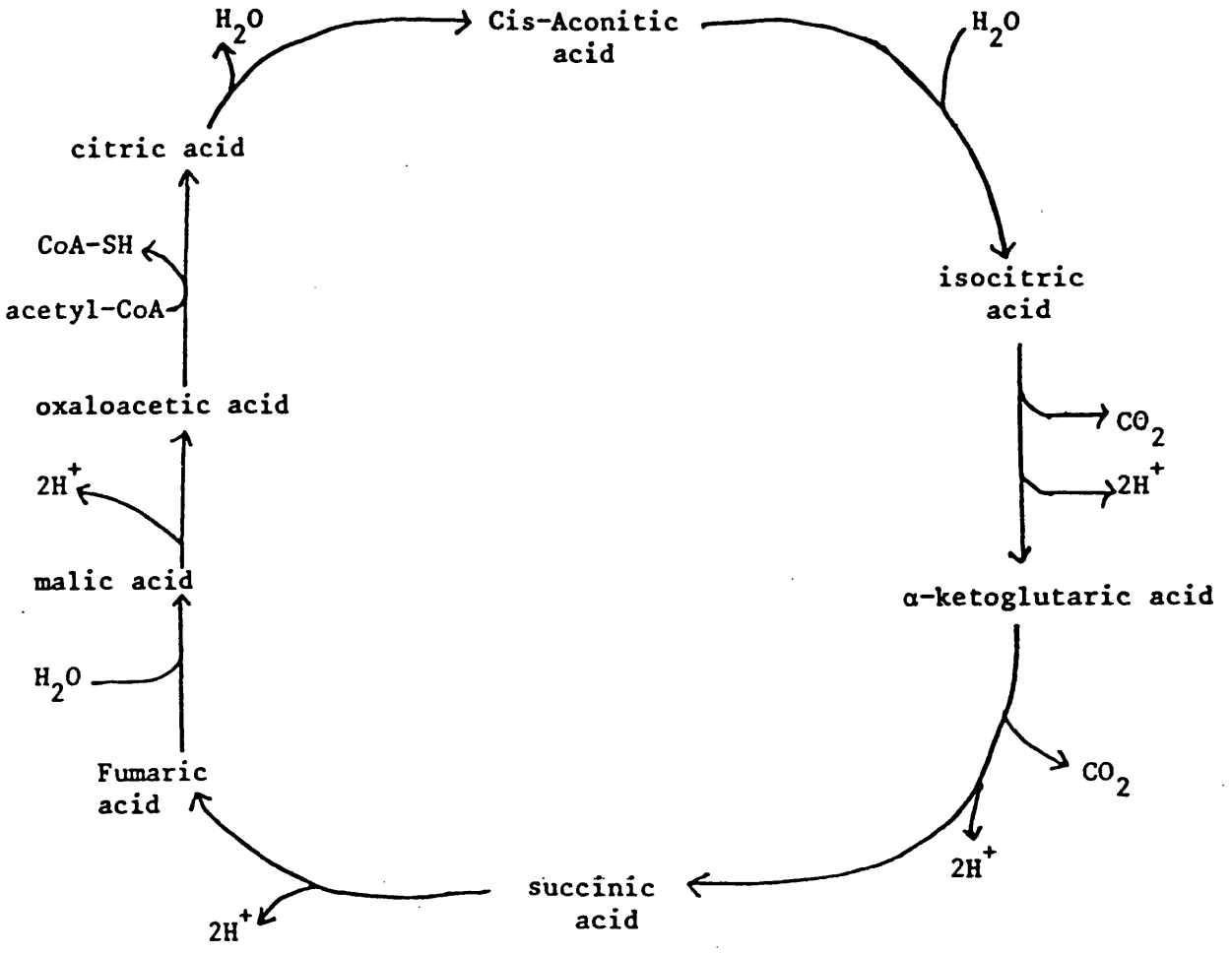
Pyruvate dehydrogenase (PDH) carries out the oxidative decarboxylation of pyruvate to acetyl CoA. The work of Randle et al (1970); Denton et al (1972) and Martin et al (1972) has shown the role of pyruvate dehydrogenase in the regulation of energy metabolism in the heart.

The acetyl CoA formed combines with oxaloacetate to form citrate and is then oxidised by the reactions consituting the tricarboxylic acid (TCA) cycle (fig 1.8). This cycle produces reducing equivalents which are oxidised by the terminal oxidation chain to yield ATP from ADP and phosphate in a process termed oxidative phosphorylation.

Pyruvate dehydrogenase and the enzymes of the TCA cycle are found in the mitochondrion. Pyruvate, therefore, has to be transported across the inner mitochondrial membrane.

Figure 1·8

The tricarboxylic acid cycle



iii. Fatty Acid Metabolism

Acetyl CoA is also the major end product of the beta-oxidation system for fatty acids, the enzymes of which are also located in the mitochondrion.

Fatty acids supplied to the heart from endogenous triglycerides or from exogenous sources are activated to coenzyme A (CoA) esters and then enter the mitochondrion by the carnitine cycle (fig 1.9) under the influence of carnitine palmitoyl transferase (CPT). This is located on both sides of the inner mitochondrial membrane (Bremer,1977). Once inside the mitochondrion the fatty acyl ester is oxidised by the beta-oxidation system (fig 1.10) to yield acetyl CoA which enters the TCA cycle as before.

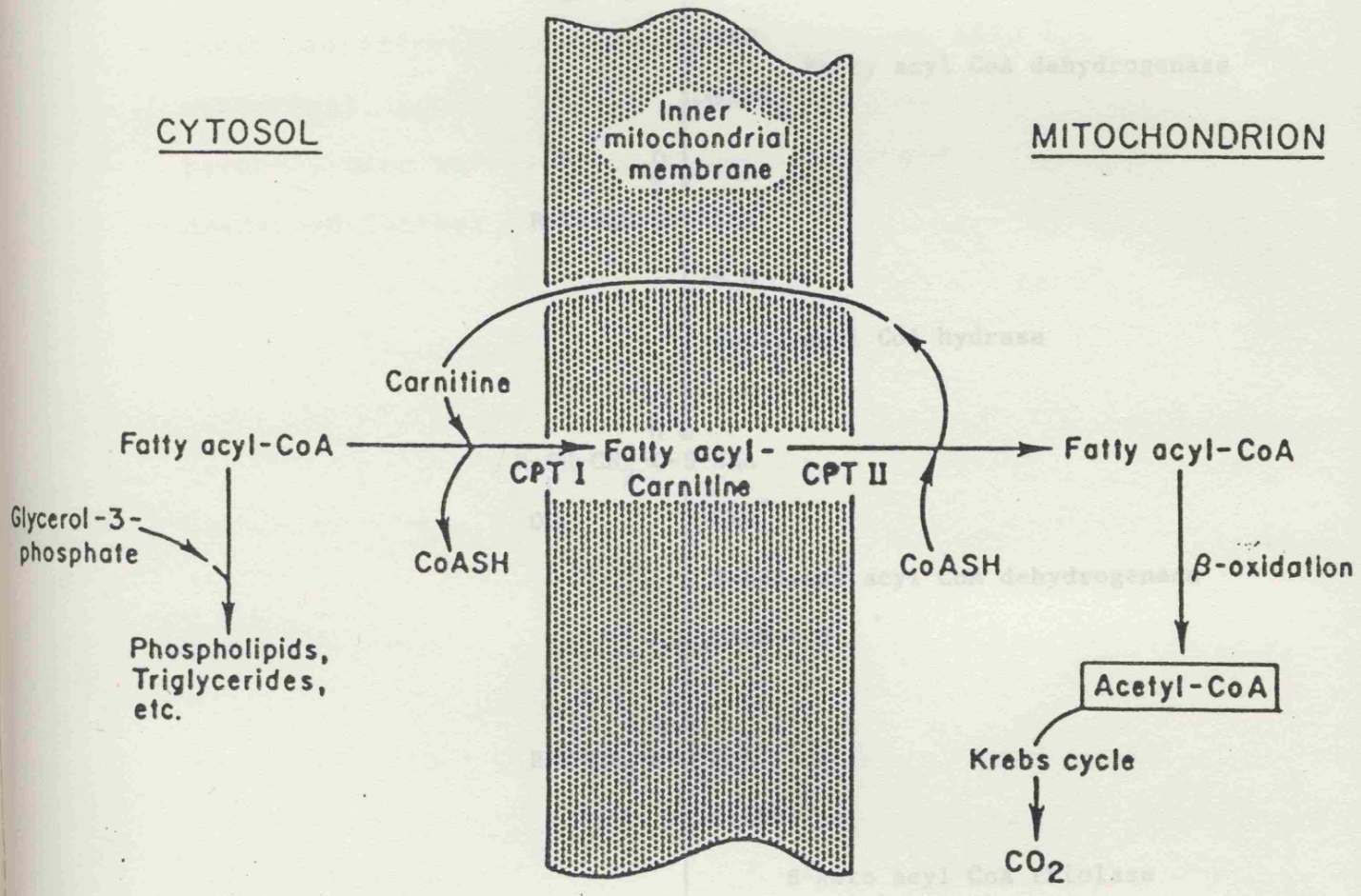
Lipid synthesis, from fatty acids, is active in the myocardium. Fatty acyl CoA esters react with sn-glycerol 3-phosphate to produce mono-, di- and mainly triglycerides (Denton and Randle, 1965). Phospholipid metabolism is believed to occur through classical routes (for review see Dawson, 1966). The production of sn-glycerol 3-phosphate from glycerol is not regarded as significant since the activity of glycerol kinase in the heart is negligible (Scheuer and Olson , 1967; Robinson and Newsholme, 1967). The major source of glycerol 3-phosphate is from glucose via glycolysis.

iv. Minor Pathways

Glucose synthesis from lactate and pyruvate can be

FIG 1·9

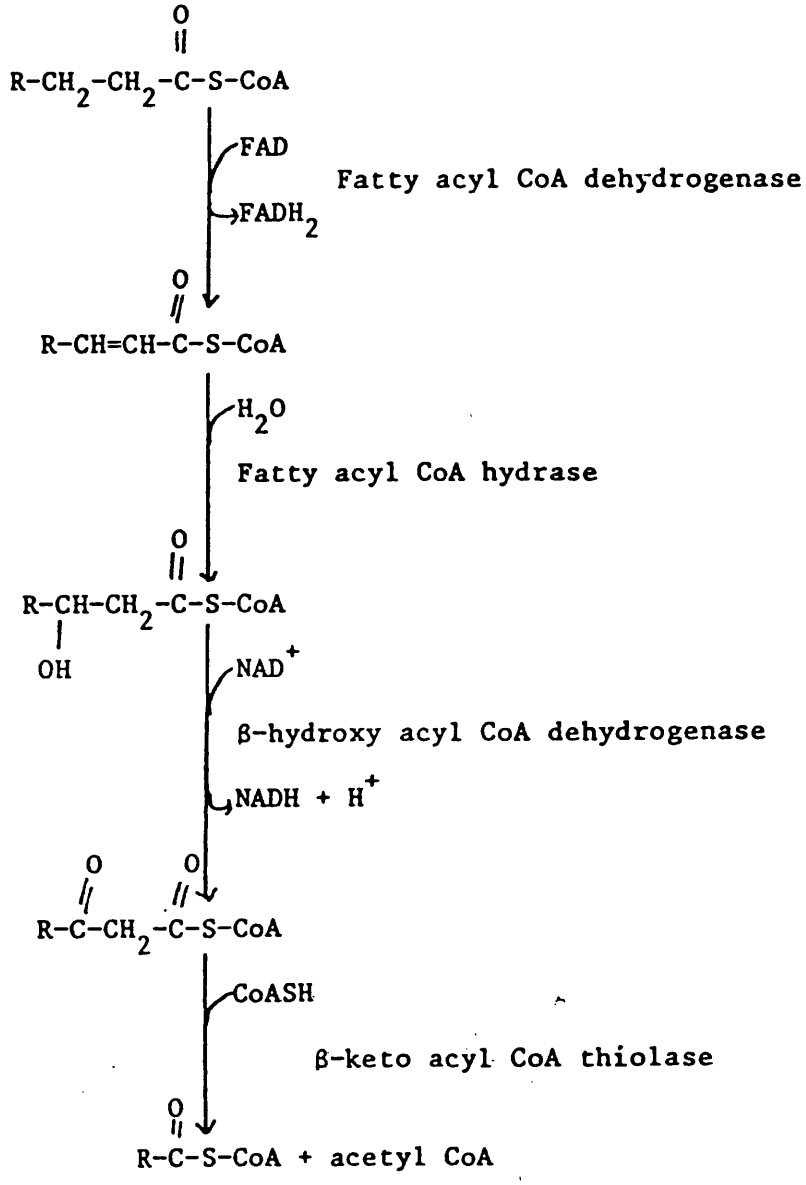
The Location of Carnitne Palmitoyl Transferase (CPT) on the Inner and Outer Mitochondrial Membrane.



from McGarry and Foster, 1980.

Figure 1·10

Reactions of the fatty acid β-oxidation pathway



observed in heart tissue (Stadie, Haughaard and Pertmultter, 1947) but the key enzymes of the Utter-Ochoa pathway (malic enzyme, pyruvate decarboxylase and phosphoenolpyruvate carboxykinase) are of low activity and the pathway is not of great significance (Racker, 1954). The same is true of the key enzymes of the hexose monophosphate shunt (glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase) as shown by Glock and McLean (1954).

Protein synthesis and degradation are obviously of great significance because of the constant need for structural, contractile and enzymic protein. This has recently been reviewed by Gevers (1984) and will not be described further here.

Section 1.5

The Heart in Ischaemia

i. General

Ischaemia is defined as the reduction of the blood flow through a tissue (or perfusate in the experimental situation). It is distinguished from hypoxia (or the extreme case anoxia) which describes a reduction of the oxygen content of blood or perfusate. In both cases the prime metabolic lesion is reduced oxygen supply but ischaemia is complicated by the reduced delivery of substrates and, more importantly, the reduced removal from the tissue of metabolic products.

Two types of ischaemia exist, chronic and acute forms. The onset of chronic ischaemia is usually characterised by a progressive narrowing of one or more of the larger coronary arteries. This occlusion results from degeneration of the arterial wall lining and gradual thickening in a process called atherosclerosis (Schettler and Boyd, 1969). Lipid and fibrous material are deposited on the intimal lining of the artery, resulting in a two-fold effect. This is primarily a reduction in blood flow, but also the artery loses its elasticity. Competent vessels expand during the peak of ventricular ejection (absorbing some of the cardiac output) and then contract during diastole to sustain diastolic flow. The significance of the loss of elasticity in coronary vessels is great since the

heart tissue is critically dependent on diastolic flow.

The heart can respond to chronic ischaemia by revascularisation and the development of anastomoses (Charlier, 1971). No such response is possible with acute ischaemia caused by either a coronary thrombosis (blockage of a vessel by a fragment of solid material, usually sloughed off from atheromatous accumulations). Or a sudden occlusion of a coronary vessel can also occur due to a spasm of the artery. The tissue affected by acute restriction of flow is rapidly depressed mechanically (within minutes) and can suffer irreversible damage within 30-60 minutes if flow is not restored (Oliver, 1972).

The effects of chronic ischaemia have recieved little experimental attention mainly due to the time required to establish this condition and the uncertainty concerning the nature of the causative factor(s). Acute ischaemia is, at present, under extensive investigation. A number of techniques are being used to study this condition. These include acute occlusion in situ of coronary vessels in dog hearts (Braunwald, Maroko and Libby, 1974), and occlusion of coronary vessels in the isolated perfused rat heart (Kannengeiser et al, 1975). Alternatively the imposition of whole heart ischaemia by the reduction of coronary flow to the whole heart (Brownsey and Brunt, 1977) can be used as a model.

ii. Mechanical Responses of the Myocardium to Ischaemia.

The onset of ischaemia is accompanied by a rapid and marked decline in the performance of the affected

tissue. In the perfused rat heart subjected to ischaemia, systolic ventricular pressure (and tension) decline, as does cardiac output, while end diastolic pressure rises (Opie, 1965; Neely et al, 1973; Kannengiesser et al, 1975 and Fisher et al, 1969).

Despite the depressed mechanical performance of ischaemic tissue, the overall performance of the heart, as shown by cardiac performance, can be maintained by compensatory mechanisms (Feola et al, 1971). These include ventricular dilation which provides increased ventricular diastolic filling and hence maintenance of cardiac output (Lekven et al, 1973).

iii. Metabolic Respones To Ischaemia

a) Carbohydrate Utilisation

One of the major responses to acute ischaemia is the shift from aerobic fatty acid oxidation to anaerobic glycolysis (Himwich et al, 1934 and Dennis and Moore, 1938). Under normal conditions the heart extracts lactate from the blood. However, in ischaemia and hypoxia a nett release of lactate is evident (Krasnov et al, 1962).

The response of the heart to ischaemia and hypoxia differ markedly. In the case of hypoxia, glucose uptake (Scheuer, 1967,72) and lactate release rise sharply. In ischaemia, although a transient rise in glucose uptake has been shown, it was maintained for only a short period (Rovetto et al, 1973). Extensive lactate accumulation occurs within the ischaemic tissue, since little capacity for

release into the perfusate is available. Subsequent inhibition of glycolysis occurs at two major sites. The decline in cellular pH would be expected to inhibit PFK activity (Kubler and Spieckermann, 1970; and Williamson et al, 1976). In addition the inability of lactate dehydrogenase to reoxidise NADH would cause inhibition of the glyceraldehyde 3-phosphate dehydrogenase reaction by reducing the availability of NAD (Rovetto et al, 1975).

b) Energy Status

In ischaemia the ATP content of myocardial cells declines more rapidly and more extensively than in hypoxia (Rovetto et al, 1973). This is likely to be a consequence of the inhibition of glycolysis during ischaemia. Not only does ATP concentration decline but a nett loss in the total adenine pool occurs as increased loss of adenosine through the cell walls has been shown (Imai et al, 1964). Reperfusion of ischaemic hearts results in a rapid resynthesis of creatine phosphate but ATP levels remain depressed (for review see Hearse, 1977) as the depleted pool of adenine nucleotides is not rapidly resynthesised.

Adenosine, arising from adenylate pool degradation, is a potent vasodilator and it also markedly decreases activated adenyl cyclase activity (Imai et al, 1964; Olsson, 1970 and Stam and DeJong, 1977)

c) Ionic Disturbances

The loss of certain ions, particularly potassium

and inorganic phosphate, has been observed from ischaemic tissue (Regen et al, 1970 and Opie et al, 1972). These disturbances are probably a reflection of the disturbed energy balance in the cell as both Na^+/K^+ ATPase and Ca^{2+} ATPase hydrolyse large quantities of ATP. The elevation of external potassium may be an important factor contributing to the high incidence of arrhythmias in severely ischaemic tissue (Brachfeld, 1973). Inorganic phosphate increases as ATP is broken down and not resynthesised (Opie et al, 1972). This ion may be a factor in the early failure of the Ca^{2+} ATPase pump due to the formation of calcium phosphate (Kubler, 1974).

d) Release of Endogenous Catecholamines

Catecholamines stored in the sympathetic nerve endings in the myocardium are released during ischaemia (Wollenberger et al, 1967). This is discussed in further detail later in the introduction.

iv. Metabolism of Fatty Acids In Ischaemia

Fatty acids and their derivatives accumulate in ischaemic tissue due to a number of factors. Both adipose tissue (Vaughan and Steinberg, 1963) and cardiac lipolysis (Kruger and Leighty, 1967) are stimulated by the presence of catecholamines, thus delivering both exogenous and endogenous fatty acids to the myocardium. At the same time, the capacity for removal of fatty acids by oxidation is depressed by oxygen limitation (Opie et al, 1973). Oxidation is inhibited at the level of beta-oxidation probably owing

to increased levels of mitochondrial NADH and $FADH_2$ (Whitmer et al, 1978). Fatty acyl CoA and fatty acyl carnitine levels also increase in ischaemia (Neely and Morgan, 1974 and Neely et al, 1972a,b).

The increase in fatty acyl CoA together with an increase in NADH (allowing production of sn-glycerol 3-phosphate from glycolytic dihydroxyacetone phosphate via glycerol 3-phosphate dehydrogenase) increases the capacity for esterification. (Neely and Morgan, 1974; Opie, 1968 and Whitmer et al, 1978). Lipid droplets have been observed in ischaemic tissue (Bryant et al, 1958 and Evans, 1964). However, Jesmok et al (1977) observed a decrease in triglyceride levels in the first 30 minutes after the onset of ischaemia followed by increased levels after 60 minutes. Whitmer et al (1978) found a much smaller increase in triglyceride synthesis than expected from the marked rise in ischaemic hearts of acyl CoA and glycerol 3-phosphate levels.

The myocardial content of free fatty acids has been variously estimated at between 30 and 10,000 nmole/g of tissue depending on the assay procedure employed, storage conditions and handling of the tissue samples (for reviews see Van der Vusse, 1983 and Victor et al, 1984). There is no evidence to suggest that under normal conditions free fatty acids are a threat for the heart. The total intracellular free fatty acid content is very low (10μmoles/g) and far below the concentration known to inhibit cytosolic and mitochondrial processes in vitro (above 50 μmoles/g) (Van der Vusse, 1983).

However, myocardial ischaemia leads to a marked

increase in tissue levels of free fatty acids up to 3-4 times the normal level (Wieshaar et al, 1977 and Van der Vusse et al, 1982). Also, derivatives of fatty acids such as acyl CoA and acyl carnitine may be harmful under ischaemic conditions. Tissue levels of acyl CoA and acyl carnitine were raised from 14 and 4 nmoles/g in the normal pig heart to 24 and 76 nmoles/g respectively in the ischaemic myocardium (Liedtke et al, 1978).

High levels of fatty acids and their derivatives cause a number of harmful effects to the ischaemic or hypoxic heart.

(1) At low concentrations (50μM) FFA labilize mitochondria and lysosomes (Acosta and Wenzel, 1974). These effects could be explained by the interference with membrane integrity. This is consistent with the hydrophobic characteristics of FFA and offers a possible general explanation for the wide range of toxic effects reported.

(2) FFA, at a concentration of 1mM, have been shown to depress contractility in the hypoxic-perfused rat heart muscle (Henderson et al, 1969 and Williebrands et al, 1973).

(3) FFA have a fairly non-specific "detergent" effect which causes enzyme inhibition at high concentrations (2μmoles/g of microsomal protein) (Pande and Mead, 1968 and Wills, 1961).

(4) At lower concentrations (50μM) FFA have been shown to specifically inhibit phosphofructokinase, a key enzyme in glycolysis (Ramadoss et al, 1976). Hexokinase, another regulatory enzyme in glycolysis, became inactivated at concentrations above 0.5mM (Lea and Weber, 1968).

(5) Both FFA and acyl CoA esters, at

concentrations known to occur in ischaemia tissue (75-100 nmoles/g dry weight), were found to inhibit mitochondrial adenine nucleotide translocase (Shug and Shrago, 1973). This would inhibit the transport of ADP into, and ATP out of, the mitochondria and further reduce energy production.

(6) In the intact tissue FFA have been argued to contrbute to the incidence of ventricular arrhythmias and death after acute myocardial infarction (Oliver et al, 1968; Julian and Oliver, 1968; Gupta et al, 1972 and Oliver, 1974). The protective effect of an anti-lipolytic agent (5-fluoro-3-hydroxymethyl-pyridine) against ventricular arrhythmias after myocardial infarction was significant provided the control of plasma FFA was good (Rowe et al, 1975). Increased lipolysis, induced by catecholamine intervention, has been shown to extend the area of ischaemic injury during acute coronary artery ligation in dogs. The converse decrease in the area of ischaemic injury by antilipolytic therapy has also been reported (Kjekshus and Mjos, 1973; Kjekshus, 1974; Lekven et al, 1973,74). Leiris et al (1975) has reported that the presence of FFA accentuates the release of lactate dehydrogenase (an index of ischaemic injury) from the isolated perfused rat heart during coronary artery ligation.

A number of reports have appeared which contradict the findings reported above. Rutenberg, Pamintuan and Soloff (1969) concluded from studies on human patients that FFA levels during the first 24 hours after infarction were not related to arrhythmias, late death or cardiogenic shock. Similarly, FFA were argued not to be arrhythmogenic in dog hearts after coronary artery occlusion <u>in situ</u> (Opie et al,

1971). In other studies FFA were found not to influence the degree of ischaemic injury produced by coronary artery occlusion in pigs (Most et al, 1974).

Although a direct link between the concentration of fatty acids in ischaemic tissue and the incidence of arrhythmias and/or the area of infarct size has not been shown, it seems likely that high levels of both tissue and plasma FFA do accentuate the deterioration of ischaemic tissue and facilitate the onset of irreversible cell damage.

Section 1.6

Lipolysis

Several discrete lipase activities have been characterized in mammalian tissues (for review see Steinberg, 1978 ; Nilsson-Ehle et al, 1980). The main activities include lipoprotein lipase (LPL), neutral triglyceride lipase (TGL), monoglyceride lipase and acid lipase. (Pancreatic lipase and intestinal lipase will not be described here).

i. Lipoprotein Lipase

This enzyme was first described by Hahn (1943) and known as clearing-factor lipase or heparin-induced lipase (for review see Robinson, 1963,70).

Lipoprotien lipase (LPL) is a glycoprotein (Chung and Scanu, 1977; Fielding et al, 1977) with a molecular weight, determined by SDS polyacrylamide gel electrophoresis of between 60,000 and 70,000 (Twu et al, 1975,76).

The enzyme is known to be present in tissue extracts of adipose, heart and skeletal muscle, lactating mammary gland, spleen, lung and kidney medulla (Robinson, 1963,70). It is not present in the brain where free fatty acids from triglycerides are not taken up from the blood by this organ.

The enzyme hydrolyses chylomicra or very low density lipoprotein (VLDL) triglyceride with the liberation of fatty acids. Fatty acids from tissue-bound LPL may be absorbed by neighbouring tissues. Alternatively, they are able to form water-soluble complexes with plasma albumin before their uptake by extra-hepatic tissue (Anderson and Fawcett, 1950; Anfinsen et al, 1952; Shore et al, 1953 and Gordon et al, 1953).

In the heart, LPL was first reported by Anfinsen et al (1952). Since then LPL has been extensively studied in both perfused and non-perfused rat heart (Robinson and Jennings, 1965; Borensztajn and Robinson, 1970a,b; Robinson, 1970; Borensztajn et al, 1973; Chohan and Cryer, 1978,79,80 and Pederson et al, 1981). Ventricular tissue appears to contain higher LPL activity (Patelsky et al, 1967). Intracellularly LPL has been found in both the microsomal (Alousi and Mallov, 1964 and Chohan and Cryer, 1979) and 100,000g supernatant (Severson, 1979) fractions. However, the functional form of the enzyme is bound to the luminal surface of capillary endothelial cells and may be released from here by heparin perfusion (Borensztajn and Robinson, 1970b and Borensztajn et al, 1975). The remaining LPL activity has been suggested to be an intracellular pool which is the precursor of the endothelium-bound LPL (Cunningham and Robinson, 1969; Borensztajn and Robinson, 1970b; Borensztajn et al, 1975; Kompiang et al, 1976; Schotz et al, 1977). Palmer and Kane (1983) have suggested that the intracellular form is active in hydrolysing intracellular triglycerides, thereby providing fatty acids for oxidation in the mitochondria.

The sensitivity of LPL to the dietary status of the animal has been reported by Robinson and French (1960); Robinson and Jennings (1965); Borensztajn and Robinson (1970a,b) and Borensztajn et al (1972). In contrast to adipose tissue LPL, the activity in the heart has been reported to increase on starvation. Refeeding animals caused a decrease in heart LPL activity while increasing adipose LPL activity (Borensztajn et al, 1972).

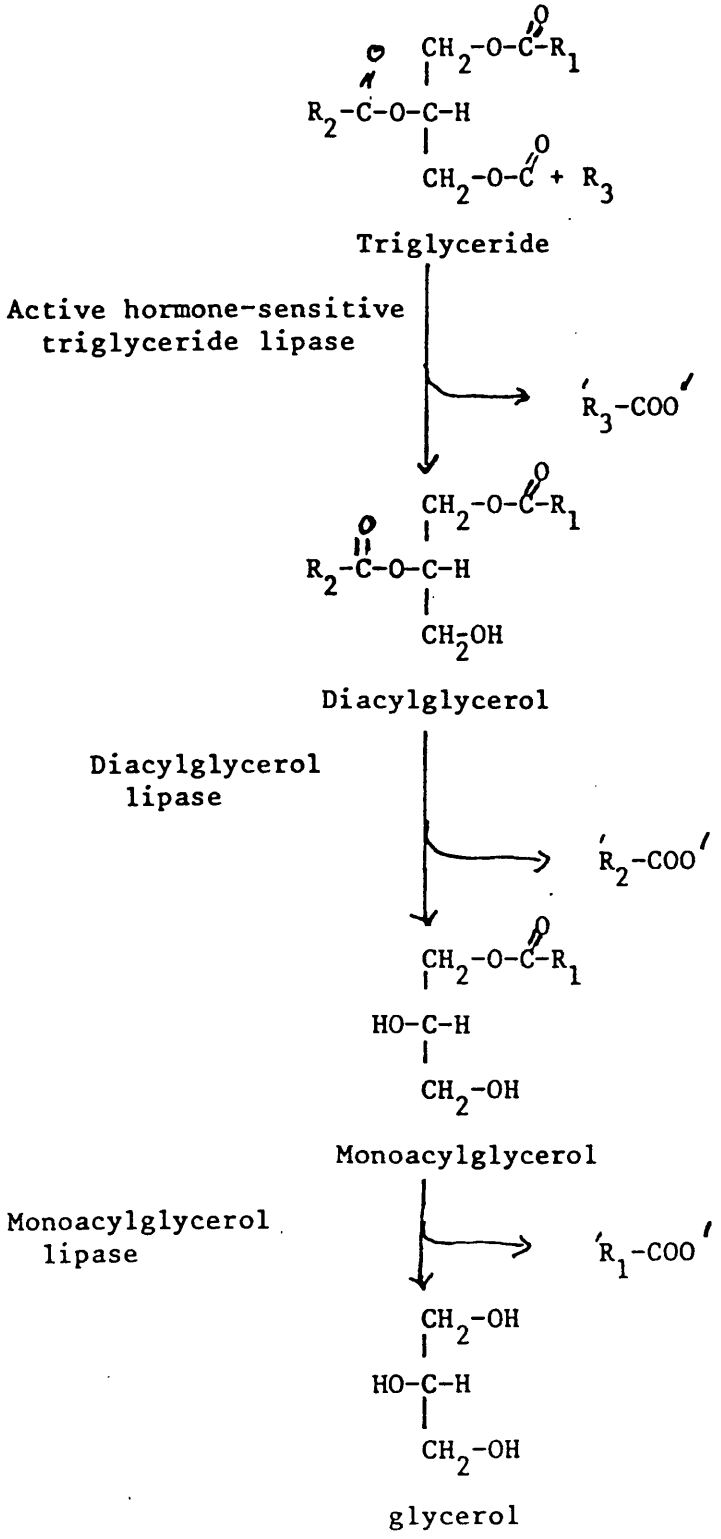
It has been suggested by Borensztajn and Robinson (1970a) and Pederson and Sholtz (1980) that the heparin-releasable (or functional) form of LPL responds rapidly to nutritional and hormonal manipulations, and it is primarily this fraction that increases in the heart and decreases in the adipose tissue during fasting.

Lipoprotein lipase preparations from various sources share the general characteristics that the enzyme is inhibited by 0.5M NaCl and protamine sulphate, and exhibits a pH optimum of 8.5. The activity is critically dependent upon the presence of serum protein cofactors, in particular apolipoprotein C_2 (for reviews see Robinson, 1970; Augustin and Greten, 1979; Severson, 1979 and Nilsson-Ehle et al, 1980).

ii. Triglyceride Lipase (Triacylglycerol lipase, EC 3.1.1.3)

Neutral triglyceride lipase (TGL) hydrolyses triglyceride to diglyceride and fatty acid (fig 1.11). The term "hormone-sensitive" lipase has often been used to refer to this enzyme as the activity has been shown to respond to

Fig. 1·11 Triglyceride hydrolysis



a number of different hormones (for review see Steinberg, 1978 and Severson, 1979).

Triglyceride lipase differs from lipoprotein lipase in a number of properties. It is not stimulated by heparin and has no requirement for serum cofactors. TGL exhibits a pH optimum of 6.8 to 7.4 and is inhibited by sodium fluoride but not by sodium chloride or protamine sulphate (Severson, 1979).

The structure of TGL is still not clearly established (Berglund et al, 1980). A partial purification of TGL from rat adipose tissue (Huttunen et al, 1970; Schwartz and Jungas, 1971 and Tsai and Vaughan, 1974) and from human adipose tissue (Pittman et al, 1972; Khoo et al, 1974 and Verine et al, 1974) has been reported. Further characterization has been limited because the enzyme appears to be lipid rich (Huttunen et al, 1970) and may well exist in more than one molecular form (Pittman et al, 1972). Several reports of the molecular weight of the enzyme have been published. As determined by SDS polyacrylamide gel electrophoresis, the enzyme from chicken adipose tissue had a molecular weight of 65,000 (Berglund et al, 1980). From rat adipose tissue it was found to be 84,000 (Khoo et al, 1980; Belfrage et al, 1980; Nilsson-Ehle et al, 1980 and Fredikson et al, 1981).

TGL activity has been localized in the microsomal, mitochondrial and lysosomal fraction of heart homogenates depending on the cell fractionation procedure or the assay conditions used (Shousboe et al, 1973; Jesmok et al, 1976; Hulsmann and Stam, 1978 and Severson, 1979). The high level of LPL activity in heart tissue may contribute to the

conflicting data. Investigators have attempted to minimize the possible contamination of TGL activity by LPL by including LPL inhibitors (NaCl and Protamine sulphate) in the assay system. However, since it is not known if these inhibitors are entirely specific for LPL (Severson, 1979), there remains doubt about such results.

Severson (1979) has claimed a clear differentiation between TGL, LPL and acid lipase activity in rat heart fractions.

iii. Diglyceride Lipase (Diacylglycerol lipase EC 3.1.1.34)

Lipase activity directed towards diacylglycerides has been shown to be present in rat adipose (Arnaud and Boyer, 1974 and Pittman et al, 1975); chicken adipose tissue (Khoo et al, 1976,78 and Berglund et al, 1980) and rat liver (Groener and Kanuer, 1981).

Because incubation with cAMP-dependent protein kinase has similar effects on the activities of both TGL and diglyceride lipase, it has been suggested that these two activities reside in the same enzyme (Belfrage et al, 1977 and Khoo et al, 1978).

iv. Monoglyceride Lipase (Monoacylglycerol lipase EC 3.1.1.23)

This enzyme has been purified from chicken adipose tissue and resolved almost completely from TGL and diglyceride lipase. The purified enzyme has been shown to be a single protein with a molecular weight of 45,000 (Berglund et al, 1980). In rat adipose tissue the enzyme has been purified by Tronquist and Belfrage (1976) and has a molecular weight of 40,000.

In the heart both diglyceride and monoglyceride lipases are reported to be present (Neely and Morgan, 1974).

v. Acid Lipase(s) (EC 3.1.1.3)

Lipase activity with a pH optimum of 4.0-4.5 has been described in rat adipose tissue (Schatz, 1965 and Hulsmann and Stam, 1978); and rabbit liver (Mahadevan and Tappel, 1968 and Guder et al, 1969); human liver (Warner et al, 1981) and rat heart (Weglicki et al, 1974; Hulsmann and Stam, 1978; Severson, 1979 and Severson et al, 1980).

The enzyme is believed to be of lysosomal origin since the lysosomal inhibitor chloroquine inhibits the acid lipase activity of cardiac and adipose tissue (Hulsmann and Stam, 1978; Severson, 1979 and Severson et al, 1980).

The acid lipase has been purified 2,500 fold from human liver (Warner et al, 1981) where it was found to have a molecular weight of 29,000. In the rat liver however, a molecular weight of 59,000 was found (Teng and Kaplan, 1974 and Brown and Sgoutas, 1980). These differences may reflect interspecies differences (Warner et al, 1981).

The substrate specificity of this enzyme has been

suggested to be triglycerides and diglycerides but not monoglycerides (Warner et al, 1981).

Section 1.7

Esterification

This section describes the enzymes of glycerolipid synthesis in mammalian tissues. The overall pathway of neutral and phospholipid synthesis from glycerol phosphate and fatty acid is shown in fig 1.12.

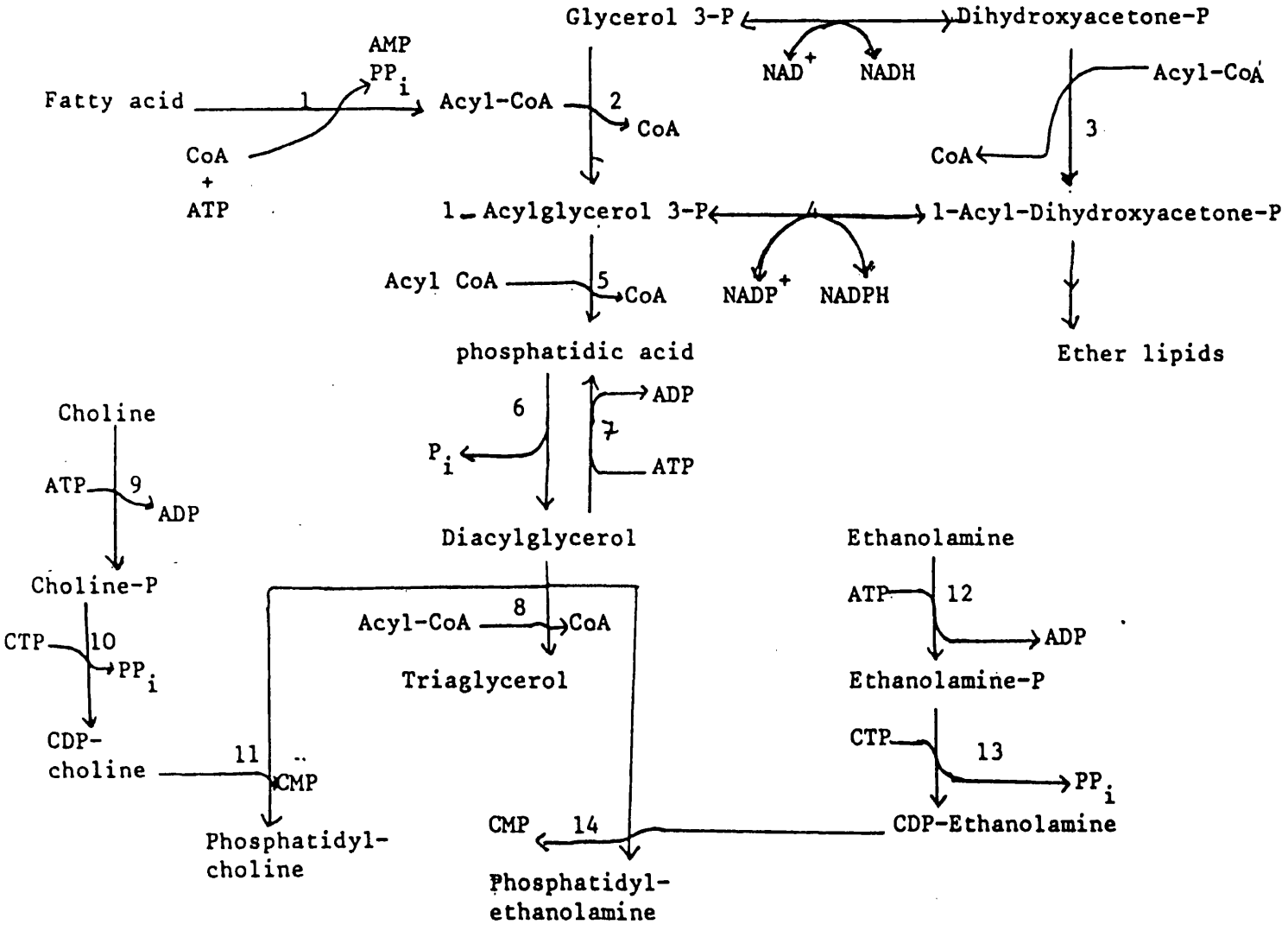
i. Fatty Acyl CoA Synthetase (EC 6.2.1.3.)

Fatty acids are activated to their respective CoA thioesters by one of several fatty acyl CoA synthetases, depending on their chain length (Groot et al, 1976). As most glycerolipids contain predominantly long chain fatty acids, long chain fatty acyl CoA synthetase will be discussed here. Long chain acyl CoA synthetase will also activate unsaturated fatty acids. It requires ATP (Pande and Mead, 1968) and is inhibited by long chain acyl CoA (Neely and Morgan, 1974). This enzyme is also known as palmitoyl CoA synthetase. It is present in most tissues (Aas, 1971; Aas and Daae, 1971; Banis and Tove, 1974; Pederson et al, 1975 and Brophy and Vance, 1976) and has been shown to be present in both the microsomal and mitochondrial fractions of liver and adipose tissue (Bloch and Vance, 1977 and Groot et al, 1976). It is closely associated with the endoplasmic reticulum and with the outer mitochondrial membrane (Garland et al, 1970; Pande and Blanchaer, 1970 and Van Tol, 1975). In the heart the enzyme appears to be located in both the

Legend for Figure 1.12

1.	Fatty acid CoA ligase	EC (6.2.1.3)
2.	Sn-glycerol-3-P-acyltransferase	EC (2.3.1.1.5)
3.	Dihydroxy acetone-P-acyltransferase	EC (2.3.1.42)
4.	Acyl (alkyl) dihydroxyacetone-P-oxidoreductase	EC (1.1.1.101)
5.	Lysophosphatidic acid acyltransferase	EC (2.3.1.-)
6.	Phosphatidic acid phosphatase	EC (3.1.3.4)
7.	Diacylglycerol kinase	EC (1)
8.	Diacylglycerol acyltransferase	EC (2.3.1.20)
9.	Choline kinase	EC (2.7.1.32)
10.	Choline-P-cytidyltransferase	EC (2.7.7.15)
11.	Diacylglycerol cholinephosphotransferase	EC (2.7.8.2)
12.	Ethanolamine kinase	EC (2.7.1.CC)
13.	Ethanolamine-P-cytidyltrasferase	EC (2.7.7.14)
14.	Diacylglycerolethanolaminephosphotransferase	EC (2.7.8.1)

Figure 1.12



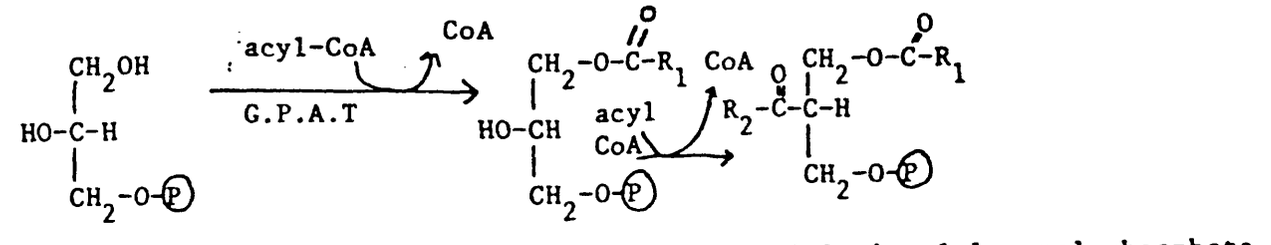
Pathways of esterification of long-chain fatty acids (enzymes of glycerolipid biosynthesis)

mitochondrial matrix (Aas and Bremer, 1968) and in the cytosol (Barth et al, 1971).

Tanaka (1979) has purified the enzyme from rat liver microsomes and mitochondria. They could not distinguish them by a large number of characteristics, and decided that there is only one form of the enzyme present in two or more locations.

ii. Sn-Glycerol 3-Phosphate Acyltransferase (EC 2.3.1.15)

The activated fatty acid acylates glycerol 3-phosphate through the acyltransferase enzymes. Two steps are involved in the acylation process.



The first involves esterification of a long chain fatty acyl group into position 1 of glycerol 3-phosphate to form 1-acylglycerol 3-phosphate. The second step involves the esterification of the 2 position to form diacylglycerol 3-phosphate (or phosphatidate).

The first step is catalysed by glycerol 3-phosphate

acyltransferase (GPAT).

GPAT has been found in both mitochondrial and microsomal fractions in rat adipose tissue and rat liver (Daae and Bremer, 1970; Kako and Liu, 1974; Jamdar and Fallon, 1973 and Kelker and Pullman, 1979). Monroy and coworkers (1972) first presented evidence suggesting that the two subcellular locations contained different isoenzymes. This has since been confirmed by others using subcellular fractionation techniques (Haldar, 1978; Nimmo, 1979; Halder et al, 1979; Yamada and Okuyama, 1978; Jamdar and Fallon, 1973 and Stern and Pullman, 1978). In adipose tissue less than 10% of the total GPAT activity is present in the mitochondrial fraction (Schlossman and Bell, 1977). In the liver and the heart the mitochondrial GPAT activity approaches that found in the endoplasmic reticulum (Daae and Bremer, 1970; Monroy et al, 1972; Liu and Kako, 1974; Zarov-Behrens and Kako, 1976; Kako et al, 1977 and Bremer et al, 1976).

In heart, liver and adipose tissue the microsomal activity is almost totally inhibited by sulphydryl reagents such as N-ethylmaleimide while the mitochondrial activity is unaffected (Haldar et al, 1979; Monroy et al, 1972 and Daae and Bremer, 1970). The difference in sensitivity to N-ethylmaliemide has been used to distinguished between the two isoenzymes (Saggerson et al, 1979).

The two isoenzymes have also been shown to differ in their Km's for palmitoyl CoA (Yamada and Okuyama, 1978) and their pH optimums (Halder, 1978). The mitochondrial enzyme has a lower apparent Km for palmitoyl CoA although such measurements are tentative when applied to lipid

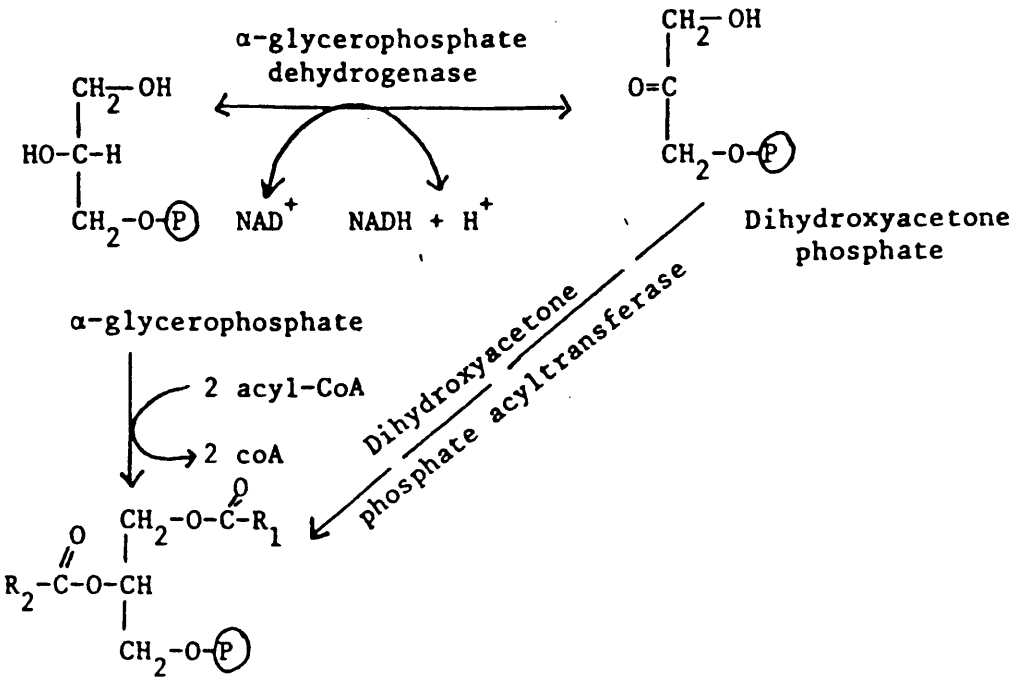
substrates since the critical micellar concentration of palmitoyl CoA is only an approximation. The Km's for glycerol 3-phosphate appear similar and in the region of 0.5mM (Tubbs and Garland, 1964; Denton and Halperin, 1968 and Evans, 1977).

It has been reported that the mitochondrial enzyme is more specific towards the fatty acyl CoA esterified than is the microsomal enzyme (Stern and Pullman, 1978). Indeed, Monroy et al (1973) found that palmitoyl CoA was esterified almost exclusively on position 1 by mitochondial GPAT. This acylation was not inhibited by oleyl CoA and linoleyl CoA. This specificity is in agreement with the observed distribution of fatty acids in naturally-occuring glycerolipids. However, it is generally accepted that the principal site of glycerolipid synthesis is the endoplasmic reticulm (Bell and Coleman, 1980); here the enzyme is not specific utilising palmitoyl CoA and oleyl CoA with equal efficiency (Halder, 1978). It appears that either extensive transacylation or desaturation must occur or that the mitochondrial enzyme is important for precise specificity during esterification. The latter hypothesis has been supported by Kelker and Pullman (1979) who found that a loss of mitochondrial GPAT activity paralleled an increase in the occurance of oleic acid at position 1 of phospholipids in cultured cells.

iii. Dihydroxyacetone Phosphate Pathway

The acylation of dihydroxyacetone phosphate and its subsequent reduction to 1 acyl:glycerol 3-phosphate provides

an alternative route to phosphatidate (La Belle and Hajra, 1974 and Hajra, 1977).



1,2-Diacylglycerol phosphate

The enzyme which catalyses the acylation of dihydroxyacetone phosphate has been shown to occur in both the microsomal and mitochondrial fractions (Schlossman and Bell, 1977,78). Schlossman and Bell (1977,78) found that the microsomal dihydroxyacetone phosphate acyltransferase is identical to the microsomal form of glycerol 3-phosphate acyltransferase. However, this has recently been disputed by Datta and Hajra (1984) who have presented evidence to indicate two separate enzymes.

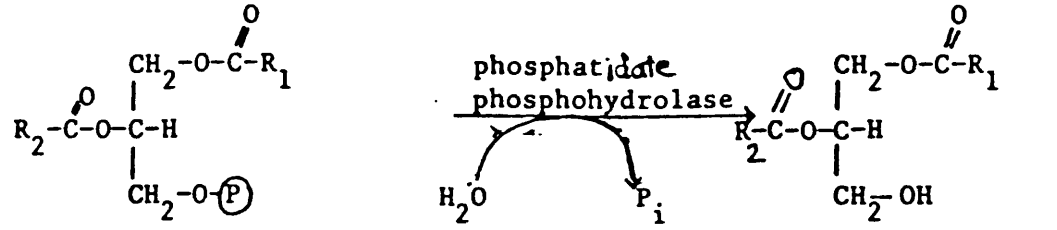
A second enzyme, initially located on the outer mitochondrial membrane (Schlossman and Bell, 1977) differs from the microsomal form in its resistance to sulphydryl inhibitors, trypsin and thermal lability (Schlossman and

Bell, 1978). Harja and his colleagues however, have located a peroxisomal site for this enzyme (Jones and Hajra, 1976,77).

The relative contributions of the glycerol 3-phosphate and dihydroxyacetone phosphate pathways to phospholipid and triglyceride synthesis remain controversial (Rognstad et al, 1974; O'Doherty, 1978 and Mason, 1978). In the heart the dihydroxyacetone pathway is said to be of minor importance (Liu and Kako, 1974).

iv. Phosphatidic acid Phosphatase (EC 3.1.3.4.)

This enzyme, also known as phosphohydrolase, catalyses the release of phosphate from 1,2 diacylglycerol 3-phosphate to form diacylglycerol.



1,2 diaclyglycerol phosphate

1,2-diacylglycerol

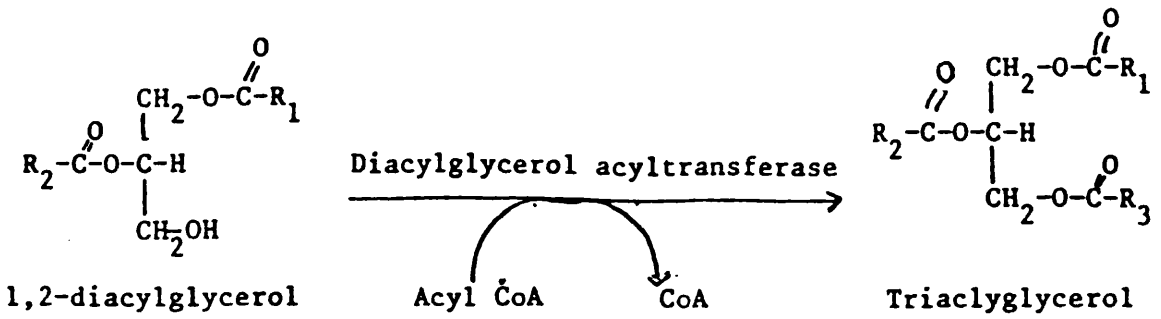
The enzyme is widely distributed intracellularly being found in mitochondria, lysosomes, microsomes and cytosol in a number of tissues (Coleman and Hubscher, 1962; Wilgram and Kennedy, 1963 and Hubscher, 1970).

The microsomal and cytosolic but not the

mitochondrial enzyme has been shown to have an obligatory requirement for Mg^{2+} ions. However, at Mg^{2+} concentrations above 5mM the microsomal, cytosolic and mitochondrial forms are all inhibited (Lloyd-Davies and Brindley, 1975). Thus there appear to be two phosphatidic acid phosphatases, Mg^{2+} independent (appearing mostly in the mitochondrial fraction) and Mg^{2+} dependent (localized in the microsomal and cytosolic fractions) (Coleman and Hubscher, 1962; Agranoff, 1962 and Caras and Shapiro, 1975).

v. Diacylglycerol Acyltransferase (EC 2.3.1.20.)

This enzyme is the only enzyme in the glycerolipid pathway unique to triglyceride synthesis. It catalyses the conversion of 1,2,diacylglycerol to triacylglycerol (Wilgram and Kennedy, 1963 and Coleman and Bell, 1976).



The investigation of this enzyme has been impeded by difficulties inherent in presenting diacylglycerol and fatty acyl CoA substrates and the extraction of the reaction products from the substrates (Coleman and Bell, 1976 and

Hosaka et al, 1977). Diacylglycerol acyltransferase from rat liver has been found to utilize a broad range of saturated and unsaturated acyl CoA thioesters containing fatty acids from C_{12} to C_{18} (Hosaka et al, 1977).

Section 1.8.

Oxidation of Fatty Acyl CoA

It is generally agreed that fatty acyl CoA thioesters within the cytosol can take essentially only two metabolic courses; either esterification to form glycerolipids or oxidation within the mitochondrion (McGarry and Foster, 1980).

It has been known for some time that the rates of esterification and oxidation in the liver vary in opposite directions under different hormonal and nutritional states (Borrebaek et al, 1976; McGarry and Foster, 1971a,b and Lossow et al, 1956). Borrebaek et al (1974) reported that, in the liver, the activity of the first enzyme in each pathway, glycerol 3-phosphate acyltransferase (GPAT) (esterification) and carnitine palmitoyl transferase (CPT) (oxidation), had a reciprocal relationship. During starvation GPAT activity rose and CPT activity fell, the reverse occurred on refeeding (Borrebaek et al, 1974). They suggested that in the liver the competing activities of the two enzymes decided the distribution of fatty acids between esterification and oxidation. However, in the heart the activities of GPAT and CPT were found not to alter with the nutritional state of the animal (Borrebaek et al, 1974). The importance of the oxidation/esterification branch point in determining the fate of fatty acids has been emphasised by a number of researchers (Fritz, 1961; Mayes and Felts, 1967; Wieland and Matschinsky, 1962; Tzur et al, 1964; McGarry and Foster, 1971a,b; Ontko, 1972,73 and Woodside and Heimberg,

1972).

i. Carnitine Palmitoyl Transferase (EC 2.3.1.21)

Carnitine palmitoyl transferase catalyses the transfer of fatty acyl CoA thioesters from the cytosol into the mitochondrion (fig 1.9.). It exists in two forms located on the outside (CPT_I) and the inside (CPT_{II}) of the inner mitochondrial membrane (Chase et al, 1965, Kopec and Fritz, 1973 and Bremer, 1977). Both enzymes have been reported to have a molecular weight of 150,000, and on treatment with sodium dodecyl sulphate dissociate into two subunits of 75,000 with a concomitant loss of activity (Kopec and Fritz, 1973). CPT_I catalyses the reaction;

palmitoyl CoA + carnitine <-----> CoA + palmitoyl-carnitine

The palmitoyl carnitine is then transferred to the inside of the inner mitochondria membrane where CPT_{II} catalyses the reverse reaction to release acyl CoA into the mitochondrial matrix (fig 1.9). The acyl CoA so formed then undergoes beta-oxidation to form acetyl CoA. It is not clear whether CPT_I and CPT_{II} activities are due to different proteins or whether their different properties (such as immunogenic differences) are due to their different presentation and position in the mitochondrial membrane (Clarke and Bieber, 1980).

The role of carnitine palmitoyl transferase (CPT) activity in determining the rate of fatty acid oxidation has

been shown in a number of studies (Fritz, 1961; Aas and Daae, 1971; Harano et al, 1972 and Norum, 1965). McGarry and Foster (1971a,b) showed that octanoic acid, a medium-chain fatty acid, which by passes the carnitine-dependent transport mechanism, was oxidised to acetyl CoA at similar rates in livers from fed, fasted and diabetic rats whose in vivo fatty acid oxidation rates were markedly different.

The view that carnitine palmitoyl transferase was the rate-limitting step for fatty acid oxidation was challenged by Van Tol and Hulsmann (1969) and Pande (1971) who found that the measurable CPT activity in rat liver mitochondria greatly exceeded that of the beta-oxidation enzymes. Subsequently it was found that malonyl CoA (an intermediate in fatty acid synthesis) was a potent inhibitor of both fatty acid oxidation (McGarry et al, 1977) and CPT activity (McGarry et al, 1978a,b) in rat liver homogenates. It was also found that malonyl CoA only inhibits CPT_I and not CPT_{II} (McGarry et al, 1978a,b). This is probably due to either the orientation of CPT_{II} in the membrane or the inability of malonyl CoA (formed by the enzyme acetyl CoA carboxylase in the cytoplasm) to enter the mitochondrial matrix (McGarry et al, 1978a,b).

Although the Ki for malonyl CoA toward CPT_I in rat liver mitochondria has been shown to be extremely low (1-2μM) the precise mechanism of ihibition has not been established. Available evidence indicates a competitive type of inhibition against long chain acyl CoA substrates (McGarry et al, 1978a,b).

The concentration of malonyl CoA in the liver is known to fluctuate with the nutritional state of the animal

(McGarry et al, 1977), and the rate of fatty acid synthesis. Thus, during starvation, fatty acid synthesis and malonyl CoA concentration are low while CPT activity and fatty acid oxidation are elevated; the reverse occurs on refeeding (for reveiw see McGarry and Foster, 1980). These changes of the rate of oxidation and esterification caused by the nutritional status appear to be mediated by the glucagon/insulin ratio (McGarry et al, 1978b). In the liver glucagon inhibits fatty acid synthesis, and thus decreases malonyl CoA concentration (Witters et al, 1979). During starvation glucagon levels are high (Borrebaek et al, 1974). On refeeding, as glucagon levels fall and insulin levels rise, malonyl CoA concentration rises as fatty acid synthesis is stimulated and CPT activity and oxidation is depressed (McGarry and Foster, 1980).

The sensitivity of CPT_I to malonyl CoA has been shown to increase with a rise in the concentration of malonyl CoA (Robinson and Zammit, 1982) and to a fall in the pH (Stephens et al, 1983).

In the heart CPT activity has been shown to be more sensitive to the concentration of malonyl CoA than the liver enzyme (McGarry et al, 1978c,83; Saggerson and Carpenter, 1981 and Saggerson, 1982). Although heart tissue does not contain a highly active cytosolic pathway for fatty acid synthesis, malonyl CoA has been found in heart tissue and its concentration is sensitive to the nutritional state of the animal (McGarry et al, 1983). Thus, a similar mechanism of CPT_I control as that in the liver but at a lower malonyl CoA concentration may apply in the heart.

There have been no reports as to the effect of acute hormonal intervention on the activity of this enzyme, either in the heart or any other tissue.

Chronic myocardial ischaemia has been shown to markedly reduce the actvity of CPT in dog hearts after 1 day of ischaemia (Wood et al, 1973). However, the effects of acute ischaemia have not been reported.

Section 1.9.

Catecholamines and the Heart

Naturally occurring catecholamines, noradrenaline and adrenaline, mediate a variety of cellular functions. They are hormonal messengers produced in response to either external or internal physiological stress and they cause changes in both the physiology and biochemistry of the animal.

In the heart, catecholamines, either from sympathetic nerve stimulation (noradrenaline) or from circulating adrenaline from the adrenal glands, cause an increase in cardiac output. This involves an increase in heart rate (chronotropy) and the force of contraction (inotropy). To meet the increased energy demands, catecholamines also increase energy production by myocardial metabolism.

Such externally acting hormones involve regulation via membrane-bound receptors. These, upon stimulation, elicit intracellular signals which mediate the cellular response of the hormone. The occurance of receptor subtypes which specifically recognise hormones has been known for some time (Ahlquist, 1948).

i. Adrenergic Receptors

It was Ahlquist (1948) who first assigned the prefixes α and β to the adrenergic receptor subtypes, based on the order of potency of a number of synthetic adrenergic agonists (stimulators) in different tissues. He concluded that there must be two kinds of adrenergic receptor. Later Lands et al (1967) using the same method as Ahlquist, showed that the β-adrenergic receptors were not homogenous but could be divided into β_1 and β_2. Noradrenaline was slightly more potent than adrenaline for the β_1 receptor while adrenaline was more potent than noradrenaline for the β_2 receptor (Lands et al, 1967).

More recently α-adrenergic receptors were found to contain two different populations (Langer, 1974). Originally, α-adrenergic receptors were found on the post-synaptic and pre-synaptic membranes of sympathetic nerve terminals and these were termed α_1 and α_2 receptors respectively (Langer, 1977,81; Starke, 1977,81 and Westfall, 1977). However, it was soon found that the preferences for appropriate and selective agonists and antagonists (blockers) upon which αreceptors had been classified, did not agree with the post- and pre- synaptic location. It has since been agreed that the terms α_1 and α_2 indicate the preference of the receptors for different agonists and antagonists and not their anatomical location (Berthelson and Pettinger, 1977; Wikberg, 1979; Starke and Langer, 1979 and Timmermans and Van Zwieten, 1981,82).

The structures of different $\alpha$and βreceptor

agonists and antagonists are shown in figs 1.13 and 1.14.

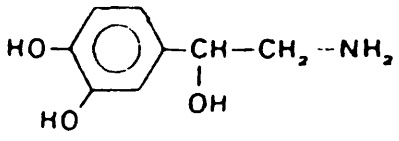
ii. Distribution of Adrenergic Receptors

The β adrenergic receptor subtypes were, according to Lands et al (1967), characterised by absolute organ specificity. However, experiments with selective β -adrenergic antagonists both in vivo and in vitro gave indication of the coexistence of β_1 and β_2 in the same organ (Carlsson et al, 1972). Recently the use of direct binding studies has led to rapid progress in the identification of adrenergic receptor subtypes (Aurbach et al, 1974; Lefkowitz et al, 1974 and Levitzki et al, 1974). Using this technique Hedberg et al (1980) found that cat and guinea pig atria contained 80% β_1 and 20% β_2 receptors while the ventricle contained almost 100% β_1 receptors. Supporting this work was the finding that noradrenaline stimulated the heart rate mainly via β_1 receptors and adrenaline stimulated the heart, at least partly, through β_2 receptors. However, the increase in the force of contraction was only evident via β_1 receptor mediated mechanisms in the ventricle of a number of mammalian species (Minneman et al, 1981 and Nahorski, 1981).

The location of the α_1 adrenergic receptor is confined mainly to the post synaptic membrane of almost all tissues innervated by sympathetic nerves (for reveiw see McGrath, 1983). All presynaptic α-adrenergic receptors appear to be of the α_2 subtype with the possible exception of the heart where both α_1 and α_2 receptors are found on the presynaptic membrane (Koblinger and Pichler, 1980,82).

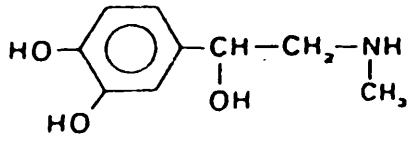
FIG 1·13

ADRENERGIC AGONISTS



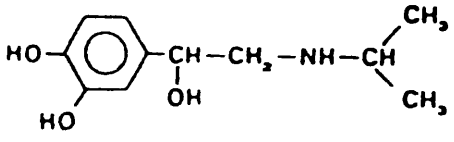
Noradrenaline
(norepinephrine)

α and β agonist



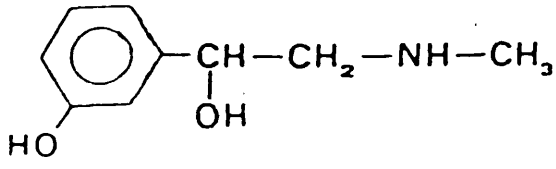
Adrenaline
(epinephrine)

α and β agonist



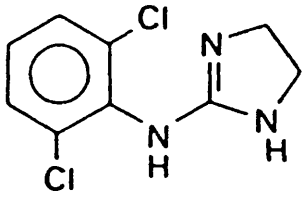
Isoprenaline

β agonist



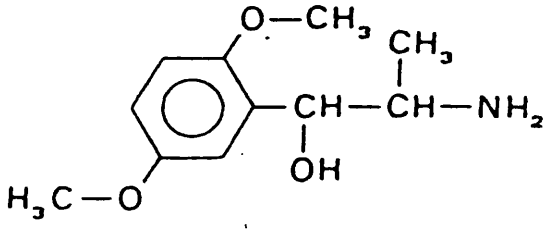
Phenylephrine

α agonist



Clonidine

α_2 agonist

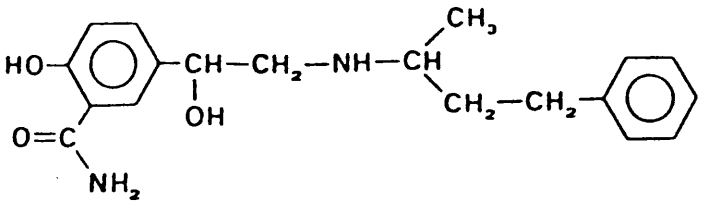


Methoxamine

α_1 agonist

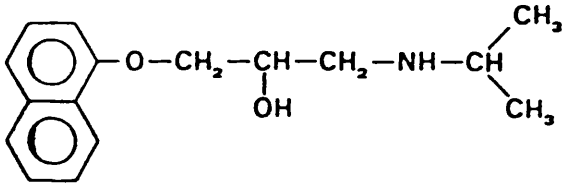
FIG 1·14

ADRENERGIC ANTAGONISTS



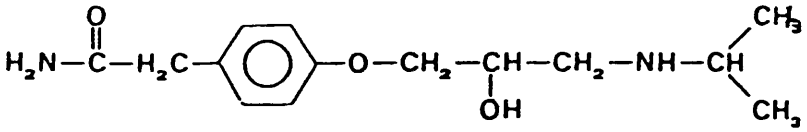
Labetalol

α and β antagonist



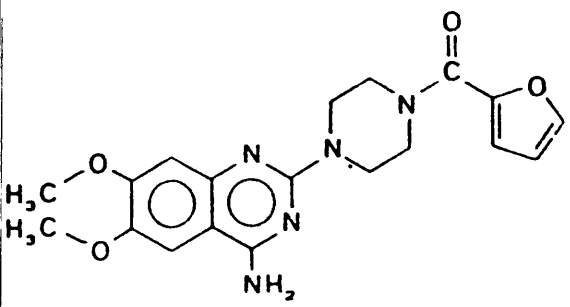
Propranolol

β antagonist



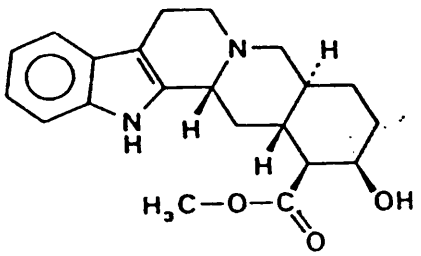
Atenolol

β_1 antagonist



Prazosin

α_1 antagonist



Yohimbine

α_2 antagonist

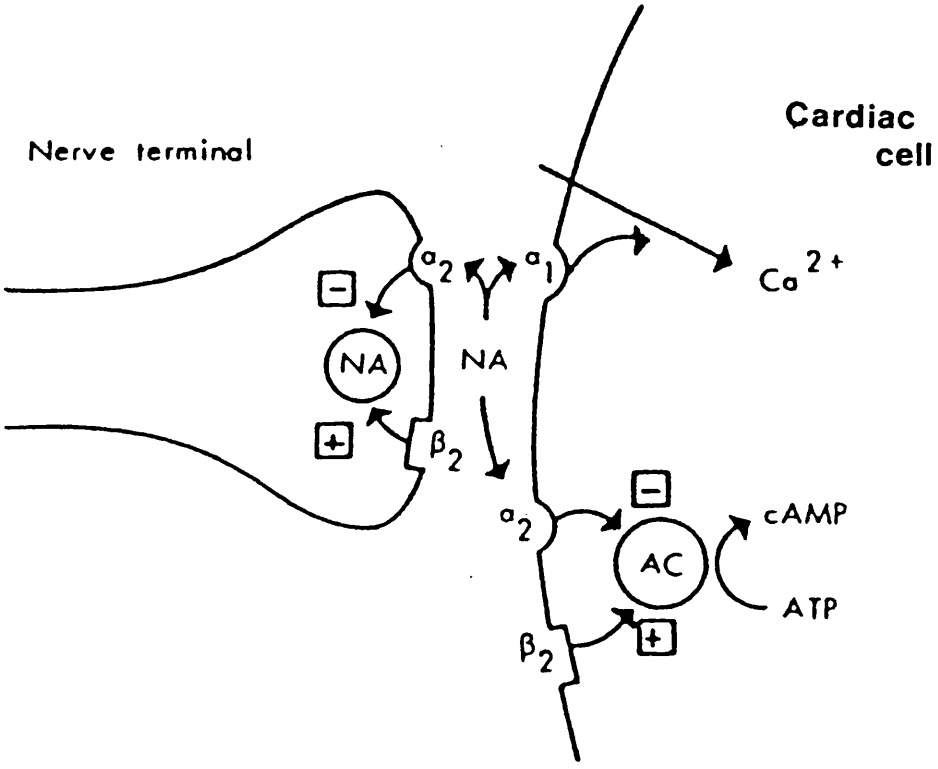
Presynaptic α_2 adrenergic receptors have been demonstrated at almost all sympathetic nerve terminals (for review see Van Zwieten and Timmermans, 1983) including the rat heart (Drew, 1979 and Pichler and Koblinger, 1978). Post-synaptic α adrenergic receptors having the pharmacological characteristics of α_2 receptors have been demonstrated in vascular smooth muscle (Berthelsen and Pettinger, 1977 and Timmermans and Van Zwieten, 1982); platelets, pancreatic islets, adipocytes (Starke, 1977); central nervous system (Schmitt, 1971; Kobinger, 1978) and in the kidney (Jarrott et al, 1979 and Summers, 1980). There has been an unconfirmed report of post synaptic α_2 adrenergic receptors in the heart (Cavero and Roach, 1980).

Drew and Whiting (1979) first reported the existence of both α_1 and α_2 receptors occurring post-synaptically in the same tissue. This has since been confirmed by other workers (Docherty and Starke, 1981; Docherty and Reichenbacher, 1981 and Timmermans et al, 1979).

Langer et al (1980) presented evidence suggesting that post-synaptic α_1 receptors are located closer to the sympathetic nerve terminal that the α_2 receptor. Adrenaline is known to be slightly more potent at α_2 than at α_1 receptors whilst noradrenaline is more potent at α_1 than α_2 receptors. Thus, it has been proposed that sympathetic nerve activity releasing noradrenaline acts mainly on α_1 receptors while α_2 mediated effects are more dependent on circulating adrenaline (Langer and Shepperson, 1982).

FIG 1·15

Distribution of Adrenergic Receptors at the Cardiac Sympathetic Nerve Terminal.



NA= noradrenaline
AC= adenyl cyclase

from Carlsson et al, 1981.

iii. Modulation of Noradrenaline Release by Presynaptic Adrenergic Receptors

Alpha adrenergic antagonists have been found to increase the overflow of noradrenaline elicited by sympathetic nerve stimulation in guinea pig and rabbit heart (Starke et al, 1972a,b). This could not be explained by decreased retention or degradation of noradrenaline within the tissue and, hence, was due to a facilitation of noradrenaline release from the sympathetic nerve terminals (Starke et al, 1971a,b). Conversely, α-adrenergic agonists, such as clonidine, phenylephrine and noradrenaline itself, inhibited the nerve stimulation-evoked release of noradrenaline (Starke, 1972a,b and Werner et al, 1970). Thus, it was proposed that the sympathetic nerve endings in the heart (and subsequently in other tissues) possess α-adrenergic receptors, the activation of which by either exogenous agonists or endogenous noradrenaline results in depression of noradrenaline release (Starke, 1971).

The release of noradrenaline is also effected by drugs with affinity for β-adrenergic receptors. In guinea-pig isolated atria, isoprenaline, a β agonist, increased, while propranolol, a β antagonist, decreased, the nerve stimulation-evoked noradrenaline release (Adler-Graschinsky and Langer, 1975). Thus, it appears that sympathetic nerve terminals also possess β-adrenergic receptors, the stimulation of which facilitates noradrenaline release (Adler-Graschinsky and Langer, 1975).

The presynaptic α receptors were shown to be of the α_2 subtype (Starke, 1981) while the presynaptic β

receptors appear to be β_2 (Westfall et al, 1970).

Presynaptic α and β receptors have been shown to occur in the hearts of a number of animals including the rat (Yamaguchi et al, 1977; Starke, 1981 and Westfall et al, 1970).

The results led to the hypothesis that the presynaptic receptors are links in local, release-controlled feedback circuits. When activated by previously released noradrenaline, they may facilitate (β_2 receptors) or depress (α_2 receptors) noradrenaline release by subsequent action potentials. This hypothesis is widely accepted for presynaptic α_2 receptors (Bacq, 1976; Starke, 1977; Stjarne, 1975; Vizi, 1979 and Westfall, 1977). However, for the presynaptic β receptors the hypothesis has not been proven (Stjarne and Brundin, 1975 and Westfall, 1977). Adrenaline is a far more potent β_2 agonist than noradrenaline and it has been proposed that the presynaptic β_2 receptors are one of the physiological targets for adrenaline (Starke et al, 1972b).

iV . Intracellular Signals Mediated by Adrenergic Receptors

After the adrenergic agonists have become bound to the receptor an intracellular signal is induced which mediates the cellular response to the extracellular stimulus. The binding of different agonists to different subtypes of adrenergic receptor has been shown to lead to the generation of different intracellular signals (for reveiw see Jakobs and Schultz, 1980).

a) Beta-adrenergic Receptors

Stimulation of either β-adrenergic receptor, ie.β_1 occuring predominantly in the heart, or β_2 occurring predominantly in vascular, bronchial and uterine smooth muscle, causes an increase in the intracellular concentration of cAMP (Cassel and Slinger, 1978; Hoffman and Lefkowitz, 1980; Lefkowitz and Hoffman, 1980; Levitzki, 1981; Levitzki and Helmreich, 1979 and Rodbell, 1980). These studies have revealed that the β-adrenergic receptor/adenylate cyclase system is composed of at least three membrane proteins: the β-adrenergic receptor, the catalytic subunit of adenylate cyclase and a guanyl nucleotide-binding protein.

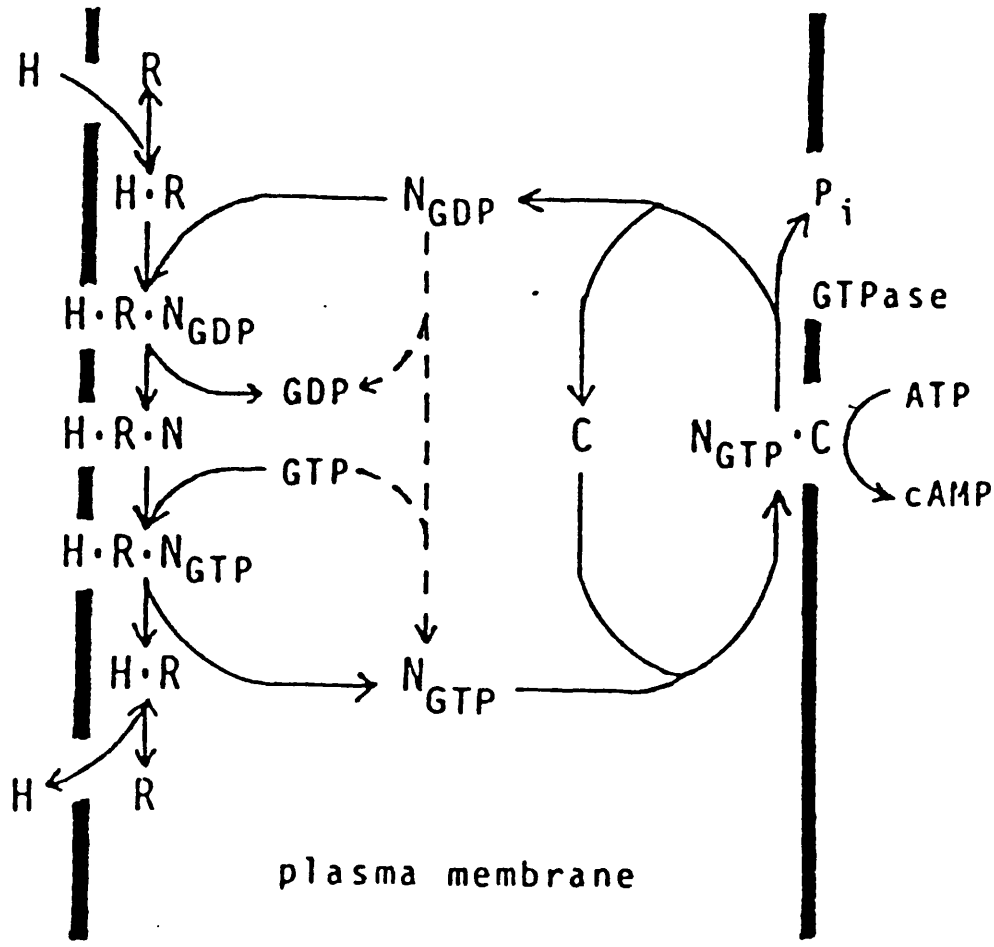
The sequence of events from binding of the β adrenergic agonist (H), to activation of the catalytic subunit of adenylate cyclase (C) by the guanylate triphosphate-activated binding protein (N) is shown in fig 1.16.

b) $Alpha_2$ Adrenergic Receptors

Binding of α_2 agonists to their respective adrenergic receptors has been shown to reduce the intracellular concentration of cAMP levels by inhibiting adenylate cyclase (Jakobs, 1979; Jakobs and Schultz, 1980 and Jakobs et al, 1980,81). Inhibition occurred almost immediatly and was reversed by appropriate antagonists

FIG 1·16

Activation of the Beta Adrenergic Receptor and the Coupling to Adenyl Cyclase Activation.



H= beta agonist
R= beta adrenergic receptor
N= nucleotide binding protein
C= adenyl cyclase

from Schultz and Jakobs, 1981.

without any lag phase (Jakobs and Schultz, 1980). Basal and hormonally-stimulated rates of cAMP formation were inhibited by between 50 and 80% (Jakobs et al, 1980,81).

The molecular mechanism by which α_2 adrenergic agonists inhibit adenylate cyclase is less clear than the mechanism of β-adrenergic stimulation of the enzyme. It appears that stimulation of α_2 adrenergic receptors increases the deactivation of adenylate cyclase by the hydrolysis of N-GTP-C to N-GDP in fig 1.16 (Arktories and Jakobs, 1981 and Jakobs et al, 1981).

Although it is known that stimulation of β -adrenergic receptors stimulate and α_2 adrenergic receptors inhibit adenylate cyclase, the basis of the different coupling of β and α_2 receptors to the enzyme is not understood. It is possible that there are different stimulatory and inhibitory guanyl nucleotide-binding proteins involved (Jakobs, 1979 and Rodbell, 1980).

c) Alpha$_1$ Adrenergic Receptors

Based on studies in a variety of tissues it is now generally accepted that the main intracellular signal generated by the occupancy of α_1 receptors is an increase in cytosolic free Ca^{2+} concentration (Berridge, 1975,80; Exton, 1981 and Michell and Kirk, 1981). Several calcium pools may contribute to this increase depending on the tissue. Besides the extracellular space, intracellular storage sites such as the mitochondria, endoplasmic reticulum and membrane phospholipids, are possible sources

of the hormone-induced increase in cytosolic Ca^{2+} in the liver (for review see Reinhart et al, 1984).

The molecular mechanisms involved in signal transformation by α_1 adrenergic receptors are far from clear. α_1 receptors are not coupled to the regulatory guanyl nucleotide-binding protein and adenylate cyclase as are β and α_2 receptors (Hoffman and Lefkowitz, 1980).

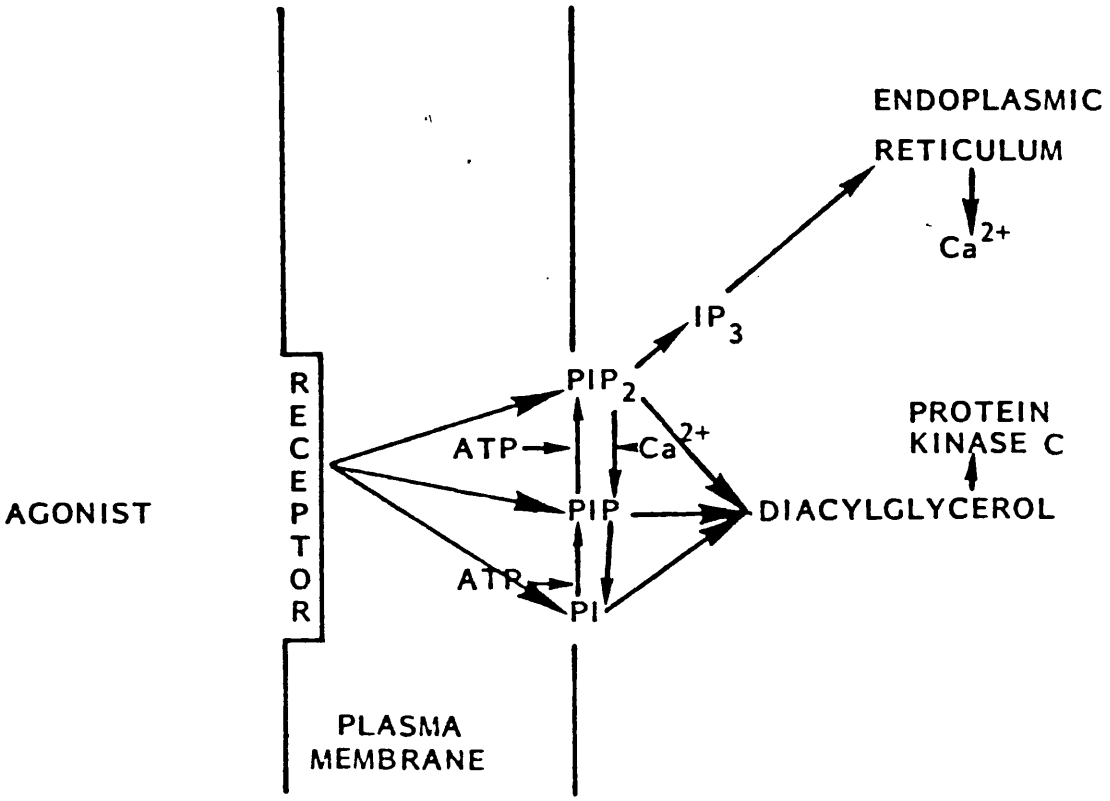
Occupancy of α_1 receptors does lead to a rapid breakdown and subsequent resynthesis of membrane phosphatidylinositol (Michell and Kirk, 1981 and for review see Fain, 1984). The production of inositol triphosphate from phosphatidylinositol 4,5-bisphosphate has been shown to increase cytosolic Ca^{2+} concentration in pancreatic acinar cells (Streb et al, 1983) and hepatocytes (see fig 1.17) (Joseph et al, 1984 and Burgess et al, 1984). Inositol triphosphate is rapidly resynthesised to phosphatidylinositol-phosphate (Litosch et al, 1983). Diacylglycerol, also formed from the breakdown of phosphatidylinositol 4,5-bisphosphate, activates protein kinase C (Kishimoto et al, 1980 and Takai et al, 1984) which phosphorylates a number of proteins (Garrison, 1983).

However, there are still large gaps in the hypothesis, eg. how inositol triphosphate increases cytosolic Ca^{2+} concentration or how membrane phosphatidylinositol turnover occurs in parallel to the increase in Ca^{2+} or even secondary to and not a causal factor (Michell and Kirk, 1981).

Other signals have been proposed for the mediation of α_1 adrenergic responses. Cyclic guanylate monophosphate (cGMP) levels have been shown to increase after α_1

FIG 1:17

Alpha $_1$ Receptor Activation



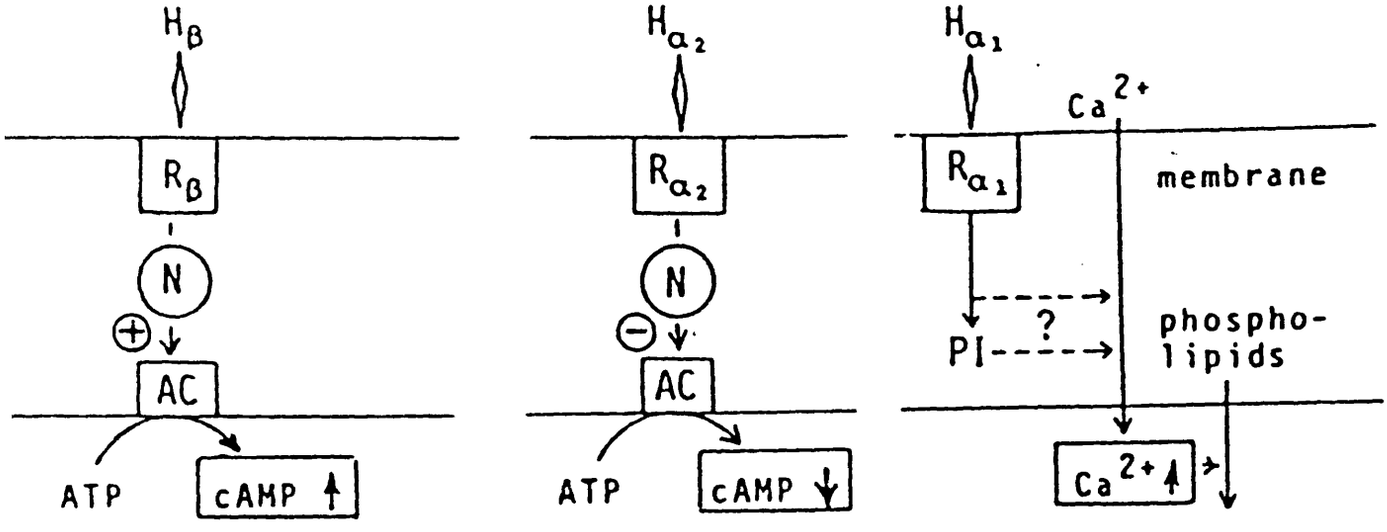
PI= phosphatidylinositol
PIP= phosphatidylinositol phosphate
PIP_2= phosphatidylinositol bisphosphate

from Fain, 1984

adrenergic stimulation in the rat ductus deferens (Schultz et al, 1975). The α_1-induced rise in cGMP levels are reduced or even abolished in the absence of Ca^{2+} (Schultz et al, 1973). It has been proposed that α_1 induced stimulation of cGMP formation involves a calcium-induced release of arachidonic acid and, possibly, other unsaturated fatty acids (Spies et al, 1980).

FIG 1:18

Intracellular Signals Iniated by Stimulation of Adrenergic Receptors



R= receptor
H= adrenergic agonist
N= nucleotide binding protein
AC= adenyl cyclase

from Schultz and Jakobs, 1981

Section 1.10.

Adrenergic Control of Lipid Metabolism

i. Adrenergic Control of Triglyceride Lipase Activity in Adipose Tissue.

Lipolysis in adipose tissue is an important part of energy metabolism in the whole animal. By regulating this process, various hormones influence the supply of free fatty acids to muscles and other tissues of the body.

Activation of lipolysis by fat-mobilizing hormones, such as adrenaline, has been explained by activation of TGL via adenyl cyclase (EC 4.6.1.1.) and cyclic 3'5' adenosine monophosphate dependent protein kinase (Butcher et al, 1965; Corbin et al, 1970 and Huttunen and Steinberg, 1971). Since the lipase is the rate-limitting step in the hydrolysis of triglyceride its activation leads to an increase in lipolysis (Vaughan and Steinberg, 1963 and Vaughan et al, 1964).

Adrenaline has been shown to increase the activity of TGL in rat epididymal adipose tissue (Rizack, 1961; Vaughan and Steinberg, 1963; Vaughan et al, 1964; Ho et al, 1967 and Huttunen et al, 1970). In 1964, Rizack showed that the TGL in fat cell homogenates could be activated under phosphorylating conditions in a reaction that was stimulated by cAMP. Since then it has been established that the general mechanism of action of cyclic AMP involved the stimulation of a protein kinase and phosphorylation of various proteins

(Walsh et al, 1968). It was proposed that a cyclic AMP-dependent protein kinase catalysed the phosphorylation of TGL and so mediated the activation of lipolysis by fat-mobilising hormones. Subsequently, TGL was found to be activated by incubation with cAMP-dependent protein kinase in rat (Huttunen et al, 1970; Corbin et al, 1970; Khoo et al, 1972 and Belfrage et al, 1977), human (Khoo et al, 1972,74) and chicken (Khoo and Steinberg, 1974) adipose tissue.

Recently direct phosphorylation of purified TGL from rat adipose tissue, by the catalytic subunit of cAMP-dependent protein kinase has been demonstrated (Belfrage et al, 1980). The extent of phosphorylation was closely correlated with the rate of lipolysis in cells stimulated with noradrenaline (Belfrage, et al, 1980).

Removal of the lipolytic hormone or addition of an antilipolytic agent reverses the activation of lipolysis (Mangeniello et al, 1971 and Allen et al, 1973). This reversibility suggests the presence of an endogenous reversible deactivator of TGL. This has been suggested to be a phosphoprotein phosphatase. Khoo and Steinberg (1974) found that a partially purified TGL from chicken adipose tissue can be activated by incubation with cAMP and muscle cAMP-dependent protein kinase, deactivated by dialysis and subsequently reactivated by addition of cAMP and ATP. This reversible activation/deactivation cycle can be repeated several times and is presumed to reflect the presence of a lipase phosphatase. Recently, direct dephosphorylation by a partially purified phosphatase has been shown to reduce activated TGL activity (Berglund et al, 1980). The

"lipolytic activation cascade" is shown in fig 1.19.

The beta-adrenergic agonist isoprenaline has been shown to increase TGL activity in rat adipose tissue and this activation was blocked by the beta-adrenergic antagonist isopropylnitrophenyl ethanolamine (Christian et al, 1969). Thus the activation of TGL by adrenaline appeared to be associated with the beta-adrenergic receptor.

Diglyceride lipase and monoglyceride lipase in adipose tissue have also been shown to be activated by cAMP-dependent protein kinase, the activation being blocked by the addition of protein kinase inhibitor (Belfrage et al, 1977; Khoo et al, 1977,78).

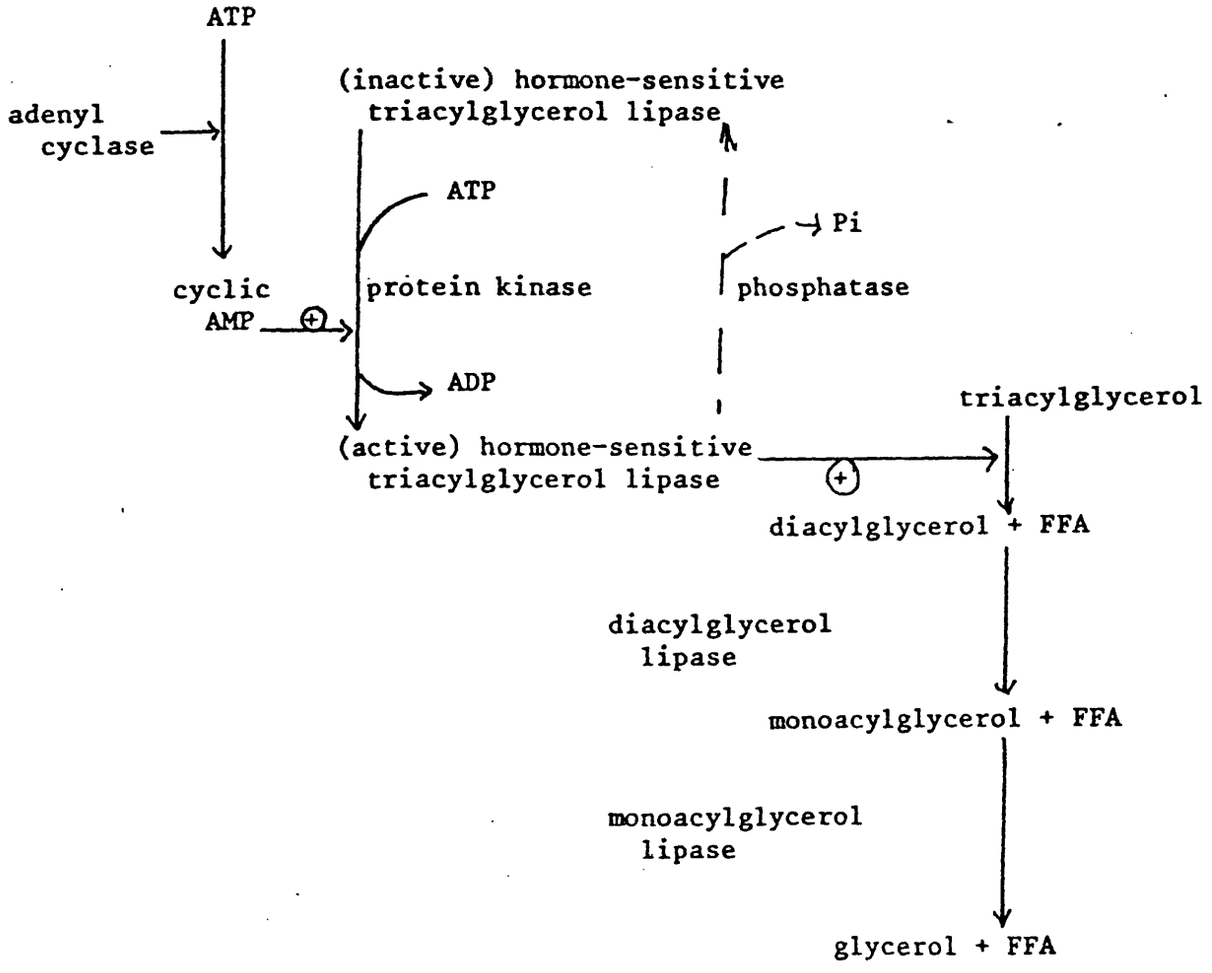
In contrast to the above proposed mechanisms, a novel system of lipolytic activation by adrenaline has been proposed by Okuda et al (1970) and Wise and Jungas (1978). These authors suggest adrenaline activation is achieved by facilitation of the substrate-enzyme interaction rather than by activation of the enzyme itself.

ii. Alpha Adrenergic Effects on Lipolysis.

It has been found that isoprenaline was over 5 fold more potent than adrenaline in activating adipose lipolysis (Fain, 1973). Subsequently, it was found that alpha adrenergic agonists inhibited lipolysis in human adipocytes (Burns et al, 1981). It has been demonstrated that alpha adrenergic stimulation reduces cAMP levels by deactivating adenyl cyclase in human and hamster, but not rat, adipose tissue (Arktories et al, 1979; Burns et al,1981). Also, lipolysis in rat brown adipose tissue has been shown to be reduced by alpha adrenergic stimulation (Sundin and Fain,

Figure 1·19

Activation of lipolysis



1983). These effects of alpha adrenergic stimulation are confined to $alpha_2$ mechanisms, stimulation of $alpha_1$ receptors appear to have little if any effect (for review Fain and Garcia-Sainz, 1983).

It is reasonably clear, therefore, that $alpha_2$ and beta adrenergic mechanisms mediate opposing effects on lipolysis in hamster and human adipose and rat brown adipose tissue. The balance between these mechanisms could be a point of regulation that determines the final effect of the natural catecholamines on adipose tissue in vivo.

iii. Adrenergic Control of Lipolysis in the Heart.

Perfusion of the heart with catecholamines increases the rate of cardiac lipolysis. This has been demonstrated by the finding of decreased tissue levels of total triglyceride (Crass et al, 1975); Gartner and Vahouny, 1973; Hron et al, 1977 and Jesmok et al, 1977) and increased rates of glycerol output (Williamson, 1964; Kriesberg, 1966; Chrisitian et al, 1969; Hron et al, 1977; Jesmok et al, 1977 and Severson et al, 1980). Cardiac lipolysis has also been shown to be increased by both adrenaline and noradrenaline when studied by the disappearance of label from isotopically pre-labelled triglyceride (Crass et al, 1975).

Adrenaline was found to increase lipase activity in heart homogenates (Bjorntorp and Furman, 1962). Similar activation of lipase activity by adrenaline was not seen by Severson (1979).

Cyclic AMP levels (Robinson and Newsholme, 1968 and Mayer et al, 1970) and the protein kinase activity ratio (Keely et al,1977; Keely and Corbin, 1975 and Hron et al, 1977) are both increased in the isolated perfused rat heart following adrenaline perfusion. As in adipose tissue this effect appears mediated by beta adrenergic mechanisms (Mayer, 1974). This would suggest that cardiac TGL could be activated by cAMP-dependent protein kinase in a way analogous to that described for adipose tissue (Shipp et al, 1973 and Hron et al, 1977). In the isolated working rat heart, the cAMP analogue dibutyryl cAMP, increased lipolysis (Crass, 1973). It also increased triglyceride mobilization in the Langendorff perfused heart (Gartner and Vahouny, 1973). However, attempts to activate cardiac TGL activity directly by incubation with cAMP-dependent protein kinase have proved unsuccessful (Severson, 1979 and Rosen et al, 1981).

Apart from hormonal control, metabolic control may also be important in regulating cardiac lipolysis. Exogenous free fatty acids are known to inhibit both basal and catecholamine-stimulated rates of lipolysis (Crass, 1973 and Crass et al, 1975). It may be that increased rates of adipose tissue lipolysis would inhibit endogenous triglyceride mobilization in the heart (Severson, 1979). Indeed, it has been shown that triglyceride levels are increased when the supply of exogenous fatty acid is elevated (Crass et al, 1971; Hron et al, 1978 and Neely et al, 1969). Severson and Hurley (1982) have shown how cardiac neutral TGL activity is inhibited by fatty acids and fatty acyl CoA compounds. Thus, the balance between synthesis and

lipolysis can be influenced not only by hormones but also by exogenous substrates.

iv. Alpha Adrenergic control of Lipolysis in the Heart.

Keely et al (1977) reported that alpha adrenergic stimulation results in a small but significant fall in cAMP levels in the perfused rat heart. This was confirmed by Watanbe et al (1977) using cardiac myocytes. However, no effect of alpha adrenergic stimulation on cardiac lipolysis has yet been reported.

Section 1.11.

Regulation of Esterification

The esterification pathway has been reported to be under hormonal control (Denton and Halperin, 1968 and Sooranna and Saggerson, 1975).

The identification of rate-limitting steps and regulatory points in the esterification pathway is, at best, tentative. Enzyme activities measured in vitro can be quite different from the in vivo situation due to the problems inherent in presenting lipid-soluble substrates to the enzymes and their intramembrane location.

Recent work by Declercq et al (1982) and Pikkukangas et al (1982) together with earlier work by Denton and Halperin (1968) have suggested that, in the liver, hormones may exert their control over the esterification pathway by altering the substrate supply. However, a large number of studies have reported direct hormonal effects on some of the enzyme activities in this pathway.

Since it is generally recognised that the endoplasmic reticulum is the major site of triglyceride synthesis, research has been concentrated on the activities of the microsomal enzymes (Numa and Yamashita, 1974 and O'Doherty, 1978).

Coleman et al (1978) have observed large increases in the microsomal forms of fatty acyl CoA synthetase, glycerol 3-phosphate acyltransferase, 1 acyl:glycerol 3-phosphate acyltransferase and diacylglycerol acyltransferase in mice fibroblasts as they differentiate

into adipocytes. These enzyme changes accompany the large increase in the capacity of these cells to synthesise triglycerides.

i. Fatty acyl CoA Synthetase

Most workers agree that fatty acyl CoA synthetase is not rate-limitting for esterification. Lloyd-Davies and Brindley (1975), using two assay methods for long-chain acyl CoA synthetase in liver found that the activity was much higher than that required to maintain maximal rates of glycerol 3-phosphate esterification.

In addition examination of enzyme activities after fasting have shown that carnitine palmitoyl transferase activity increases while glycerol 3-phosphate acyltransferase activity decreases in the liver (Borrebaek et al, 1974). Since carnitine palmitoyl transferase (McGarry and Foster, 1980) and glycerol 3-phosphate aclytransferase (Lloyd-Davies and Brindley, 1975) have been cited as regulatory points for oxidation and esterification respectively, it seems unlikely that fatty acyl CoA synthetase, the preceeding enzyme for both pathways, can be a rate-limiting step for either pathway (Bell and Coleman, 1980).

However, in adipose tissue, where esterification plays a far more dominant role, the situation may be different. Indeed insulin has been shown to increase (Jason et al, 1976) and adrenaline to decrease (Sooranna and Saggerson, 1978) the activity of long-chain acyl CoA synthetase.

ii.Glycerol 3-phosphate acyltransferase

It has long been thought that the activity of this enzyme could be the major regulatory point for the esterification pathway (Denton and Halperin, 1968). A number of direct hormonal effects on the activity of this enzyme have been shown and these will be discussed later.

iii. Phosphatidic acid Phosphatase

This enzyme has also been cited as a major regulatory point in triglyceride synthesis (Lamb and Fallon, 1974 and Lawson et al, 1981). Lamb and Fallon (1974), Fallon et al (1977) and Brindley et al (1979) report evidence suggesting that nutritional and hormonal alterations in the rate of esterification are paralleled by changes in the activity of this enzyme.

Acute hormonal effects on the activity of phosphatidic acid phosphatase have also been reported (Cheng and Saggerson, 1978) and evidence for a phosphorylation/dephosphorylation system of control has been presented (Berglund et al, 1980). However, since the enzyme is not the first unique step in the biosynthetic pathway it is unlikely to be the sole rate-limiting step for glycerolipid synthesis. The variations reported with diet and hormones has been disputed by Murthy and Shipp (1980).

iv. Diacylglycerol acyltransferase

This enzyme is the only one exclusively concerned with the synthesis of triglycerides and thus, may be considered as a potential regulatory point (Bell and Coleman, 1980).

Diet-induced changes in triglyceride synthesis in adipose tissue were paralleled by marked changes in the activity of this enzyme (Fallon et al, 1975). Sooranna and Saggerson (1978) have reported a stable decrease in diacylglycerol acyltransferase activity after fat cells were exposed to adrenaline. Recently, Haagsman et al (1981) have shown that the activity of this enzyme can be inactivated by cAMP-independent phosphorylation and can be reactivated under dephosphorylation conditions.

Diacylglycerol acyltransferase activity has been claimed to have the lowest activity of all the enzymes in triglyceride synthesis in both the rat and rabbit heart (Kako and Patterson, 1975 and Murthy and Shipp, 1980) and thus, it may be rate-limiting in triglyceride synthesis in the heart.

v. Adrenergic Control of Glycerol 3-phosphate Acyltransferase Activity (GPAT).

This enzyme is the first, and probably major, regulatory point in the regulation of glycerolipid synthesis (Bell and Coleman, 1980). Acute hormonal effects on the activity of this enzyme have been observed by a number of workers.

Sooranna and Saggerson (1976,78) have shown that

exposure of either adipose tissue or isolated fat cells to adrenaline or noradrenaline results in a marked decrease in GPAT activity. This effect was prevented by inclusion of the beta blocker propranolol or insulin (Sooranna and Saggerson, 1978). The effect of noradrenaline was seen on both GPAT activity from the mitochondrial (N-ethylmaleimide insensitive) and from microsomal (NEM sensitive) fractions (Rider and Saggerson, 1983a).

Soler-Argilaga et al (1978) reported a decrease in esterification after livers were perfused with the cAMP analogue, dibutyryl cAMP, and suggested the involvement of glycerol 3-phosphate acyltransferase. Nimmo and Houston (1978) presented evidence to indicate that the activity of GPAT in liver microsomes was inactivated by a cAMP-dependent phosphorylation mechanism and could be reactivated by a phosphatase. Recently, however, Rider and Saggerson (1983a,b) were unable to confirm these reports. They suggested that the inactivation, by noradrenaline, of the mitochondrial and microsomal forms of the enzyme seen in rat adipocytes was due to a protein present in the post-microsomal supernatant, and similar to an acyl CoA binding protein.

There have been no reports on the role of alpha adrenergic mechanisms in the control of esterification or GPAT activity.

Very little is known about the regulation of esterification in the heart. It has been demonstrated that the concentration of triglycerides in the heart is reduced as a result of adrenaline perfusion (Gartner and Vahouny, 1973). This could be a result, not only of increased

lipolysis, but also of decreased esterification.

Section 1.12.

Catecholamines in the Ischaemic Heart

i. Release of Endogenous Catecholamines During Ischaemia.

Several investigations have indicated that increased adrenergic activity occurs in the acutely ischaemic myocardium (Shahab and Wollenberger, 1967; Shahab et al, 1969; Abrahamsson et al, 1981; Corr et al, 1978; Holmgren et al, 1981 and Podzuwiet et al, 1978). This could be due to an increase in nerve impulse flow in the heart, either by activation of sympathetic nerves (Karlsberg et al, 1979 and Staszewska-Barczak, 1971) or by local cardiac reflexes (Brown and Malliani, 1971 and Bosnjak et al, 1979). In addition, direct effects on adrenergic neurones (independent of nerve impulses) could all play a part in increasing noradrenaline release or decreasing re-uptake (Langer, 1980). Certain prostaglandins, for example, prevent excessive stimulation of adrenergic receptors by inhibiting noradrenaline release (Langer, 1980 and Malik, 1978), while a high extracellular K^+ concentration seen during ischaemia can increase noradrenaline release (Lorenz and Vanhoutte, 1975). The fall in pH in the ischaemic myocardium soon after coronary occlusion would be expected to inhibit noradrenaline release (Puig and Kirpekar, 1971) and re-uptake (Karapati et al, 1974). Hypoxia (Shahab and Wollenberger, 1967) and energy deficiency within the neurone itself (Wakade and Furchgott, 1968) could both play a part

in increasing noradrenaline release.

Although a large volume of evidence indicates an increased release of noradrenaline in the ischaemic heart, a number of reports have contradicted this (Marshall and Parratt, 1976; McGrath et al, 1981 and Riemersma and Forfar, 1982). These workers have suggested that the increase in noradrenaline overflow from the ischaemic area seen in earlier reports (Shahab et al, 1972) occured during reperfusion and not during occlusion. However, absence of noradrenaline overflow during ischaemia should not be interpreted as absence of noradrenaline release from nerve terminals as the major fate of released noradrenaline is normally re-uptake into the neurones (Iverson, 1977).

In acute ischaemia cAMP levels are known to rise markedly (Dobson and Mayer, 1973; Krause et al, 1978; Podzuweit et al, 1978; Wollenberger et al, 1969 and Opie et al, 1979). Occlusion of the left coronary artery in the isolated rat heart model for 2 minutes resulted in a marked increase in cAMP levels in both the non-ischaemic and ischaemic areas (Krause and Wollenberger, 1967). After 20 minutes of occlusion cAMP levels had returned to normal in the non-ischaemic area but remained elevated in the ischaemic area. Concurrent with the rise in cAMP levels Krause and Wollenberger (1967) found an increase in the activities of cAMP-dependent protein kinase, phosphorylase kinase and phosphorylase in ischaemic tissue. Pre-perfusion before occlusion by the beta adrenergic antagonist propranolol completely prevented the rise in cAMP levels and the activity of cAMP-dependent protein kinase but only partly prevented the rise in phosphorylase kinase and

phosphorylase activity in ischaemic tissue (Krause and Wollenberger, 1967).

ii. Arrhythmias Associated with Ischaemia and Reperfusion

Abnormalities of the heart beat which occur during ischaemia or subsequent reperfusion account for at least half of the deaths due to acute myocardial infarction (Armstrong et al, 1972). These abnormalities, incuding ventricular arrhythmias, ventricular fibrillation, ectopic beats and supra-ventricular arrhythmias, are often associated with an increase in plasma catecholamine levels (Gazes et al, 1959; Valori et al, 1967; Siggers et al, 1971; Webb et al, 1972 and Videbaek et al, 1972).

There is impressive evidence incriminating myocardial noradrenaline in the development of ventricular arrhythmias during acute myocardial ischaemia and reperfusion. Surgical denervation of the heart significantly reduces the incidence of ventricular fibrillation following coronary occlusion providing that it is carried out long enough prior to the occlusion to allow depletion of myocardial catecholamines to occur (Ebert et al, 1968). Chemical denervation by 6 OH-dopamine almost completely abolished the occurance of ventricular fibrillation during both coronary occlusion and reperfusion in the cat (Sethi et al, 1973 and Sheridan et al, 1980) and guinea pig (Culling et al, 1984) heart.

iii. Pre-perfusion with Beta Adrenergic Antagonists

There are numerous reports concerning the effects of β-adrenergic antagonists on the functional, electrophysiological and morphological changes in the heart following acute myocardial ischaemia (Fitzgerald, 1972). In the dog model alone there are 27 reports concerning the effects of six different β antagonists on cardiac arrhthymias and survival after acute ischaemia. The conclusion from such studies is that β-adrenergic antagonism will;

(1) increase the time before ventricular arrhthymias occur (Rosenfeld et al, 1978);

(2) reduce the incidence of arrhythmias (Khan et al, 1972) and

(3) improve survival (Evans et al, 1976).

However, there have been 3 reports where β antagonism with propranolol has not reduced the incidence of arrhthmias or the survival rate (Pearle et al, 1978; Pentecost and Austen, 1966 and Khan et al, 1972).

In the isolated perfused rat heart model, Lubbe et al (1978) showed that adrenaline increased tissue cAMP levels and lowered the electrophysiological threshold for ventricular fibrillation. These changes were prevented by adding atenolol, a cardio-selective β_1-adrenergic antagonist, to the perfusate. Following ligation of the left coronary artery in the same preparation, there was a fall in ventricular fibrillation threshold and a rise in cAMP levels. This was not blocked by atenolol but was blocked by

the non-selective β antagonist propranolol (Lubbe et al, 1978).

Acute ligation of the left coronary artery in rats causes a predictable pattern of ventricular arrhythmias in about 60% of animals (Kenedi and Losonci, 1973). The effects of various β adrenergic antagonists in this model have been assessed by a number of studies (Kane et al, 1979; Campell and Parratt, 1981; Szekeres, 1978 and Siegmund et al, 1979). The various studies clearly indicate that β-adrenergic antagonism has a beneficial effect on the consequences of occlusion of the left coronary artery in rats.

iv.Pre-perfusion with Alpha-adenergic antagonists.

The α-adrenergic antagonist phentolamine has been shown to reduce the incidence of ventricular arrhythmias caused by nicotine or adrenaline (Leimdorfer, 1953); acontine or chloroform inhalation (Vargaftig and Coignet, 1969); digitalis-induced arrhythmias (Ettinger et al, 1969) and also in man following acute myocardial infarction (Gould et al, 1971+75).

In a recent study a chloralose-anaesthetised cat model of coronary occlusion and reperfusion was used to study the relative importance of α and β adrenergic receptor blockade (Sheridan et al, 1980). These workers found that either α blockade with phentolamine or depletion of endogenous noradrenaline with 6 OH-dopamine significantly reduced the incidence of ventricular arrhythmias during both

coronary occlusion and reperfusion. In contrast, the β antagonist propranolol only reduced the incidence of arrhthmias during occlusion and not during reperfusion. It was also found that prasozin, a specfic α_1 adrenergic antagonist gave the same results as phentolamine, thus conferring α_1 specificity to the beneficial effects of antagonists (Sheridan et al, 1980).

Preliminary evidence has suggested that α receptor responiveness may be altered during myocardial ischaemia (Moore and Parratt, 1973). Shayman et al (1980) have suggested that the number of α receptors is increased in the early phase of reperfusion while Sheridan et al (1980) give evidence of an enhanced α-adrenergic responsiveness soon after reperfusion of the ischaemic area has begun.

v. Ischaemia, Catecholamines and Lipid Metabolism

The increased release of noradrenaline may play a significant role in the development of tissue damage during ischaemia. It is believed to contribute, not only to the incidence of ventricular arrhythmias, but, by increasing metabolic disturbances, it causes myocardial cell damage (Corr and Gillis, 1978; Hjalmarso, 1980 and Maroko et al, 1971). Several workers have shown that depletion of catecholamines from the heart (Gaudal et al, 1979; Gercken and Doring, 1973 and Sakai and Spieckermann, 1975) or β -adrenergic antagonism (Sakai and Spieckermann, 1975; Manning et al, 1980; Naylor et al, 1980 and Pieper et al, 1980) have been beneficial in protecting against hypoxic or ischaemically induced myocardial injury.

One of the possible causes of cell damage and consequent myocardial dysfunction is the cellular accumulation of long chain fatty acids and their metabolites resulting from catecholamine-stimulated lipolysis of endogenous triglyceride (Opie, 1976). High levels of fatty acids and their metabolites have been implicated in the genesis of arrhythmias in the infarcting myocardium (Oliver, 1974) while antilipolytic agents have been shown to reduce infarct size and protect against ventricular arrhythmias (Ilebekk and Mjos, 1973; Ilebekk and Lekven, 1974 and Rowe et al, 1975). Thus, the regulation of lipid metabolism in the ischaemic heart is relavent to the occurance of arrhythmias and the extent of myocardial damage which can occur.

METHODS AND MATERIALS

Section 2.1

Perfusion of Hearts

(For source see Materials).

i. Perfusion apparatus.

Hearts were perfused by a non-recirculating Langerdorff system. The apparatus is shown in fig 2.1. The perfusion system consists of water-jacketed glassware connected by polythene tubing (internal diameter 0.86 mm, external diameter 4.8 mm). The perfusate was pumped by a variable speed peristaltic pump (Watson Marlow MHRE 22, Falmouth, Cornwall). The silicon tubing at the peristaltic pump measured 3.2 mm (i.d.) and 6.4 mm (o.d.).

Air bubbles were removed from the system by the bubble trap. A thermostated-recirculating pump supplied heated water from a reservoir to the water-jacketed glassware. The perfusate also passed through a heat exchanger shortly before entering the heart. The temperature of the perfusate emerging from the cannula was 37^{o}C. The perfusate was filtered before entering the heart by a 25 mm diameter prefilter and filter (0.45μm pore size) and aerated by flowing downwards through a jacketed reflux column against a flow of humidified 95% O_2 / 5% CO_2.

To calibrate the flow rate the perfusate leaving the cannula was collected in a measuring cylinder over a 2

Components of the perfusion apparatus

A heart perfusion vessel

C variable speed peristaltic flow inducer

D bubble trap

E temperature equilibration coil

F membrane filter and housing

G cannula

H perfusate aerator column

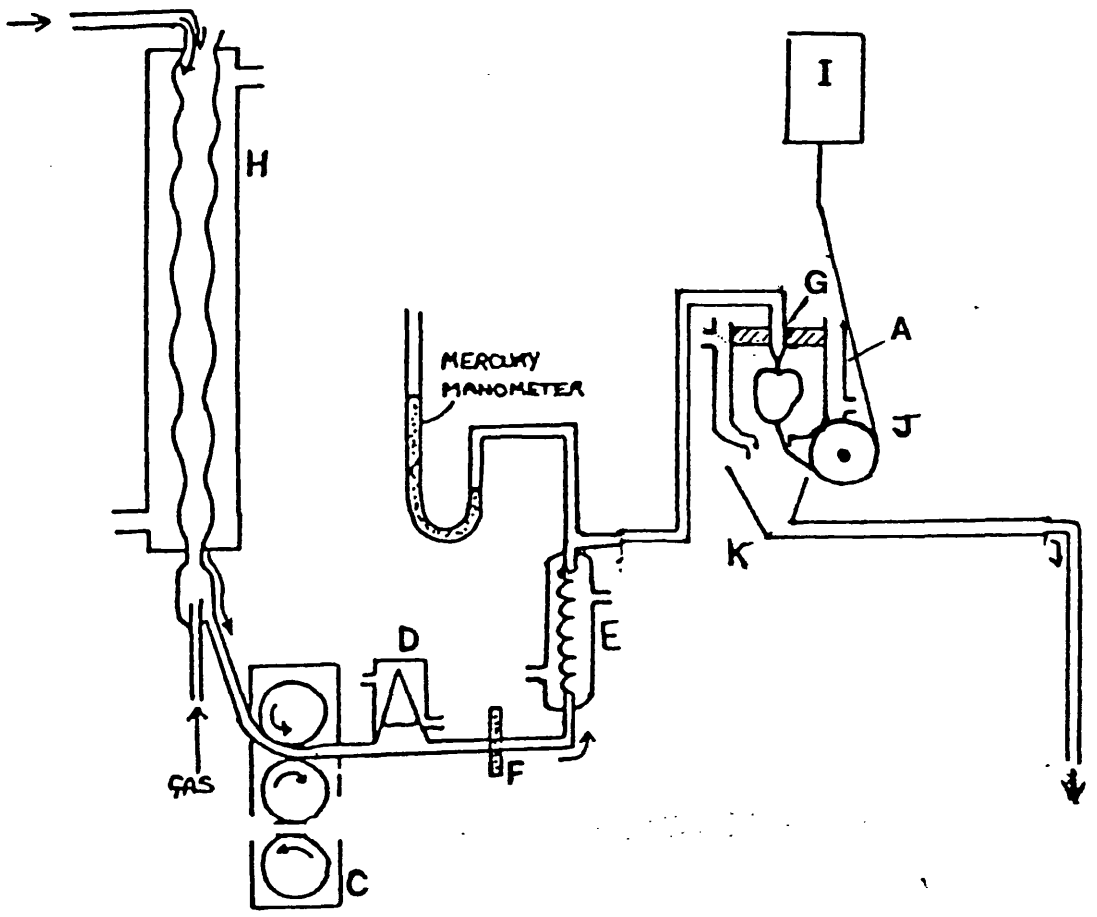
I strain gauge

J wheel

K collecting funnel

Fig. 2.1

Perfusion apparatus



minute period. For all perfusions a flow rate of approximately 10 ml per minute was used. The total volume of the perfusion apparatus was 15 ml.

ii. Preparation of Perfusate

The perfusate was glucose bicarbonate buffer (Krebs and Hensleit, 1932) pH 7.4. The final composition of the Krebs/Hensleit solution was: sodium chloride (118.4 mM), potassium choride (4.6 mM), sodium hydrogen carbonate (24.9 mM), magnesium sulphate (1.1 mM), sodium dihydrogen orthophosphate (1 mM) and calcium chloride dihydrate (2.54 mM). To prevent the precipitation of calcium phosphate, all the salts except calcium chloride were dissolved in double distilled water and the solution gassed for 20 minutes with 95% O_2/ CO_2. The calcium chloride, dissolved in distilled water, was then added to the buffer. Immediately before use, glucose was added to a final concentration of 11 mM.

iii. Perfusion procedure.

Male albino rats, Wistar strain, weighing 200g and fed ad libitum on laboratory chow (Oxoid 41B) were used. The rats were anaesthetised by intraperitoneal injection of a mixture of sodium pentobarbitone ("Sagatal", May and Baker, Dagenham, Essex) 80 mg/kg, and heparin (sodium salt, Evans Medical, Speke, Liverpool), 24,000 U/kg. To prepare the anaesthetic, 58 mg of heparin was dissolved in a mixture of 5.2 ml of Krebs/Hensleit buffer and 0.6 ml of Sagatal. 0.8

ml of this preparation was introduced into the rats by intraperitoneal injection.

The heart was quickly excised and placed in ice-cold Krebs/Hensleit buffer. This cold buffer arrested the heart beat. Blood was rinsed free of the heart while extraneous (mainly adipose tissue) material was removed. The aorta was attached to a vinyl cannula with an artery clip. (The cannula was made from an intravenous cannula, o.d. 1.65 mm, cut obliquely 3.5 mm from the head). The heart was then mounted on the perfusion apparatus and immediately perfused with Krebs/Hensleit buffer and glucose at 37^{o}C. The aorta was tied to the cannula with a silk suture ("Abra Silk", black braided, no.2).

A thread was attached to the apex of the heart by a small hook. The thread was attached to a UF2 dynamometer strain gauge by way of a pulley wheel. The physiological recorder (Devices Instruments Ltd., Welwyn Garden City, Herts) recorded total and developed tension of the heart after resting tension had been set at 2 grammes. Heart rate was measured manually using a stop watch.

After 20 minutes of perfusion, hearts were removed from the cannula, placed in ice-cold Krebs/Hensleit buffer and the atria and aorta transected from the ventricles. The ventricular muscle was then homogenized in 5 ml of ice-cold STE buffer (0.25 M sucrose, 10 mM Tris, 1 mM EDTA, pH 7.6) for 10 seconds by a polytron homogeniser (Northern Media Supply Ltd., North Cave, North Humberside) at a power setting of 3.5.

iv. Perfusion Procedure for the study of the Affects of Adrenergic Agonists and Antagonists.

For the investigations of the affects of adrenergic agonists the perfusion was continued for 20 minutes before the agonist was introduced in to the system, via the reflux column, to a final concentration of 10^{-6}M. The peak effect of any change in inotropy or chronotropy was noted 1-2 minutes after the addition. At this time the hearts were removed and homogenised as before.

When investigating the affects of adrenergic antagonists, hearts were perfused normally for 10 minutes and thereafter with perfusate containing the antagonist (10^{-6}M) for a further 10 minutes. At this time the hearts were removed and homogenized as before.

v. Perfusion Procedure for the study of the Affects of Coronary Occlusion and Reperfusion.

Hearts were perfused normally for 10 minutes, after which a silk suture was inserted behind the left descending coronary artery using a small surgical needle. The artery was occluded by ligation of the silk suture. The ischaemic area of the left ventricle was clearly visible after 10 minutes occlusion. At this time hearts were removed and placed in ice-cold Krebs/Hensleit buffer. Tissue samples (approximately 100mg) were quickly dissected from the ischaemic area of the left ventricle and the non-ischaemic area of the right ventricle. These were homogenised

separately in 3 ml of ice-cold STE buffer.

For reperfusion studies a polythene tube (id 0.86 mm, od 1.52, length 4 mm) was inserted between the artery and the suture just before ligation. This was removed after 10 minutes occlusion, allowing reperfusion of the artery. Tissue samples were taken from previously ischaemic and non-ischaemic areas, as before, at 1 minute and 5 minutes after reperfusion had begun.

When using the adrenergic antagonists the procedure for coronary occlusion and reperfusion was followed. The antagonist was introduced into the perfusate 10 minutes before occlusion and remained present throughout the rest of the perfusion at a concentration of 10^{-6}M.

To study the affects of 6-hydroxydopamine rats were injected intravenously 24 hours before use. 50 mg of 6 OH-dopamine was dissolved in 1 ml of Krebs/Hensleit buffer. This was injected into the rats to give a concentration of 50 mg/ml. The following day the procedure to study the affects of ischaemia or of reperfusion were followed.

vi. Sterilization and Washing of the Perfusion Apparatus.

To avoid and bacterial contamination the apparatus was filled with an antibiotic mixture of penicillin (300 U/ml), streptomycin (1mg/ml) and neomycin (1mg/ml). This solution remained in the apparatus overnight. After approximately 20 perfusions the apparatus was disconnected and the components washed first in chromic acid then in Decon 700 detergent. (Decon Ltd., Hove). This was followed

by a wash with distilled water. At the same time all the vinyl tubing and three way taps were replaced.

Section 2.2.

i. Extraction of Enzyme.

Fresh tissue was immediately homogenised in the polytron homogenizer (SM20 generator, power setting 3.5) for 10 seconds in STE buffer. The tube was maintained cold by immersing it in an ice bath. The homogenate was then either used directly as a crude glycerol 3-phosphate acyltransferase (GPAT) preparation or it was centrifuged at 2,000g in a bench centrifuge at 4°C for 5 minutes. This supernatant was either used directly as a crude triglyceride lipase (TGL) preparation or it was centrifuged at 10,000g in an Eppendorf microfuge at 4°C for 8 minutes. The pellet so formed was resuspended in STEB buffer (0.25 M sucrose, 10 mM Tris, 1 mM EDTA, and 0.1% fatty-acid poor bovine serum albumin, pH 7.6) to give a final protein concentration of approximately 2 mg/ml. This was used as a mitochondrial preparation for the assay of carnitine palmitoyl transferase (CPT) and the mitochondrial form of GPAT. The 10,000g supernatant was used as a crude preparation of the microsomal form of GPAT.

ii. Incubation with N-ethylmaleimide (NEM).

200µl of homogenate was incubated in the presence or absence of 15 mM NEM for 5 minutes at 30°C. This was then assayed for GPAT activity. NEM-sensitive activity was calculated as that due to the microsomal form of the enzyme. NEM-insensitive (mitochondrial) activity was calculated as the difference in activity between that measured in the absence and presence of NEM.

iii. Sonication of Mitochondrial Pellet.

400µl of mitochondrial suspension (2 mg/ml of protein) was sonicated for two periods of 15 seconds at a power setting of 40 watts using a sonicator (Model 180, Ultrasonics Ltd.) The mitochondrial suspension was kept cool throughout by immersion in an ice bath. Sonicated suspensions were measured for total carnitine palmitoyl transferase activity and compared to normal mitochondrial suspensions.

Section 2.3

Enzyme Assays

i. Glycerol 3-phosphate acyltransferase (GPAT).

This method is derived from the work of Evans (1977). The principle of the method is:

a) Long chain acyl CoA + glycerol 3-p $\xrightarrow{\text{GPAT}}$ monoacyl glycerol 3-P + CoA

b) Long chain acyl CoA + monoacyl glycerol 3-P $\xrightarrow{\text{Mgpat}}$ diacyl glycerol 3-P + CoA.

MGPAT = monoacyl glcerol 3-P acyltransferase and is not rate-limitting.

This assay was performed at 30°C in a final volume of 100µl containing 0.25 mM sucrose, 10 mM Tris, 1 mM EDTA, 50 mM potassium chloride, pH 7.4 with 5 mM [U-^{14}C] glycerol 3-phosphate (ammonium salt) (0.1µCi), 100 µM palmitoyl CoA and 0.2 mg of fatty-acid poor bovine serum albumin. The reaction was initiated by the addition of 10 µl of homogenate (approximately) 50µg of protein). After 5 minutes the reaction was stopped by the addition of 300µl of TCA-saturated butanol. After vortexing the layers were separated by centrifugation (10,000g) in an eppendorf microfuge for 2 minutes. The aqueous layer was removed while

the butanol layer was washed with 750µl of butanol-saturated trichloroacetic acid (TCA). After a further centrifugation a 100µl sample of the butanol layer was dissolved in 2 mls scintillation fluid (600 ml Triton, 10g 2,5- diphenyloxazole (PPO), 1400 ml toluene). Each time point was done in triplicate. A zero time point was also measured and subtracted from the measurement at 5 minutes. Radioactivity was counted in a Tricarb liquid scintillation counter (Packard Instruments Ltd., Caversham, Berks). GPAT activity is expressed as nmole of glycerol 3-phosphate esterified per minute per mg of protein.

ii. Carnitine Palmitoyl Transferase (CPT) (2.3.1.2.1.)

This method is a modified version of the method employed by Saggerson (1982). The princple of the method is as follows:

Palmitoyl CoA + L(-) Carnitine $\xrightarrow{CPT}$ Palmitoylcarnitine + CoA.

The assay was carried out at 30°C in a final volume of 100µl containing 0.25M sucrose, 10mM Tris, 50mM potassium chloride, 1mM EDTA, pH 7.4; with 0.5mM DL-[methyl-^{14}C]-carnitine hydrochloride (0.05µCi), 50µM palmitoyl CoA and 0.02mg of fatty-acid poor bovine serum albumin. The reaction was initiated by the addition of 20µl of mitochondrial suspension (approximately 40µg of protein). After 3 minutes the reaction was stopped by the addition of 50µl of ice-cold 6M HCl. 300µl of water-saturated butanol was then added. After vortexing, the layers were separated by centrifugation in an Eppendorf microfuge for 2 minutes. The aqueous layer was removed while the butanol layer was washed with 750µl of butanol-saturated water. After vortexing and recentrifuging a 100µl sample of the butanol layer was dissolved in scintillation fluid. This was done in triplicate and a zero time-point was subtracted from the average. Radioactivity was counted in a Tricarb Liquid scintillation counter.

iii. Triglyceride Lipase Assay

The activity of triglyceride lipase was determined by a modified version of the method of Severson (1979).

The Glycerol tri [1-^{14}C] oleate (triolein) substrate, 9.2mM in hexane (specific activity 1.6μCi/μM) was dried under nitrogen and resuspended in an equal volume (3.125 ml) of absolute ethanol. A solution of 264mg of triolein in 1ml hexane was prepared and then 0.1ml of this was added to 3.025ml of absolute ethanol. This was used to increase the concentration of triolein in the radioactive aliquots. 10μl of "hot" triolein and 20μl of "cold" triolein were mixed together just before use.

The incubation was carried out in a total volume of 80μl. Final concentrations were 0.4mM ^{14}C triolein (0.15μCi), 50mM sodium phosphate and 0.08mg of fatty-acid poor albumin pH 7.5. This suspension was sonicated for 30 seconds immediately before use. The reaction was initiated by the addition of 10μl of supernatant (approximately 40μg of protein) and incubations proceeded for 30 minutes at 30^{o}C. The assay blank contained 10μl of water or buffer in place of the supernatant. All incubations were performed in triplicate. The assay was stopped by the addition of 300μl of an extraction solution containing methanol:chloroform:heptane (1.41: 1.25: 1) and 0.1mM carrier oleic acid (Belfrage and Vaughan, 1969). 10μl of 1M sodium hydroxide was then added. The mixture was vortexed vigorously and then centrifuged in an Eppendorf microfuge

for 3 minutes. An aliquot (50µl) of the upper aqueous phase was dissolved in 4ml of scintillation fluid containing 5µl of 1M HCl and counted in a Tricarb liquid scintillation counter.

iv. Removal of Fatty Acid From Bovine Serum Albumin

Bovine serum albumin fraction V (Sigma Chemical Co., Poole Dorset), was defatted according to the method of Chen (1967). 50g of albumin was dissolved in 500ml of water at 4°C . 25g of activated charcoal was added, the pH adjusted to 3 with 1M HCl and the mixture stirred at 4°C for 1 hour. The charcoal was removed by centrifugation at 20,000g for 20 minutes at 4°C. The charcoal pellet was washed with 100ml of acidified water (pH 3, 4°C), recentrifuged and the supernatant fractions pooled. The supernatant was filtered through a 50mm diameter 0.45µm pore size membrane filter (to remove the remaining charcoal), the pH readjusted to 7 with 2M sodium hydroxide and the solution was freeze-dried. The fatty-acid poor albumin was stored at -20°C.

Section 2.4

i. Purification of cAMP-Dependent Protein Kinase.

Cyclic-AMP-dependent protein kinase was prepared

from rabbit skeletal muscle by the method of Walsh et al, (1968).

1.1kg of rabbit skeletal muscle was homogenised in a Waring Blender at low speed at 4°C for 45 seconds with 2.5 l of 4mM EDTA. All the following steps were also performed at 4°C. The suspension was centrifuged at 6,000g for 45 minutes and the supernatant decanted through glass wool. The supernatant was adjusted to pH 6.1 with 1M acetic acid and the precipitate formed was collected by centrifugation at 6000g for 45 minutes and discarded.

The supernatant was adjusted to pH 6.8 using 1M dipotassium hydrogen phosphate (pH 7.2). Fractionation was performed by the addition of 32.5g of ammonium sulphate per 100ml of supernatant. The precipitate, collected by centrifugation (20,000g for 45 minutes) was dissolved in 150ml of 5mM dipotassium hydrogen phosphate, 2mM EDTA, pH 7 and dialysed against 3 changes of 5l of the same buffer. The protein solution was then centrifuged at 80,000g for 1 hour and the precipitate discarded.

Whatman DE52 anion exchange (Whatman Ltd., Springfield, Maidstone, Kent) was equilibrated with 5mM dipotassium hydrogen phosphate, 2mM EDTA, pH 7 and poured into a column (24cm x 4.5cm). The protein solution was run onto the column followed by 300ml of 5mM, 20mM, 50mM, and finally 100mM dipotassium hydrogen phosphate, pH 7 all containing 2mM EDTA. The eluate was collected in 10ml fractions and assayed for protein spectrophotometrically at A280. Fractions from the same peak containing 0.4mg/ml of protein or higher were pooled. Ammonium sulphate was added to the pooled samples at 0.4mg/ml and the precipitate so

formed was collected by centrifugation (20,000g for 45 minutes) and redissolved in 3 mls of 5mM dihydrogen sodium phosphate, 2mM EDTA pH 7. The samples were then assayed for the activity of cAMP-dependent protein kinase.

Two peaks of protein kinase activity were found. The first eluted with 20mM phosphate, the second with 100mM phosphate. The former activity was used in subsequent incubations to study the effect of cAMP-dependent protein kinase on the activity of glycerol 3-phosphate acyltransferase and triglyceride lipase.

These were stored in 100µl aliquots of the phosphate buffer at -25°C.

ii. Preparation of the Catalytic Subunit.

Whatman DE 52 anion exchange was equilibrated with 50mM dipotassium hydrogen phosphate, 2mM EDTA, pH 7 and poured into a 5ml column. The cAMP-dependent protein kinase holoenzyme was equilibrated with the same buffer. 1ml of the enzyme solution was run on to the column. 10mls of 50mM K_2HPO_4, 2mM EDTA, ph 7 and 10µM cAMP was then run through the column and the fractions containing the catalytic subunit were pooled. This was concentrated by dialysis against 70% w/v sucrose and dialysed further against 50mM K_2HPO_4, 10mM mercaptoethanol and 3% glycerol. This was kept in the fridge (4°C) for up to one month.

iii. Cyclic AMP-dependent Protein Kinase Assay

A final volume of 100µl contained 60mM sodium dihydrogen phosphate, 20mM sodium fluoride, 2mM magnesium chloride, 1mM EGTA, pH 7.5, with 300µM Adenosine 5'-γ[^{32}P] triphosphate, triethylammonium salt (approximately 50cpm/pmol), 30µM cyclic AMP and 0.5mg of histone (Sigma, type IIIS). The reaction was initiated by the addition of approximately 50µg of enzyme protein (25µl). At intervals from 30 seconds up to 5 minutes duplicate samples of 10µl from the reaction mixture were spotted on to Whatman filter paper (No.1) squares and placed in 10% trichloroacetic acid (TCA). 30 minutes after the last addition the TCA was replaced by a fresh volume of TCA. After a further 30 minutes the filter paper squares were washed with ethanol, dried and placed in scintillation vials with 3ml of water. These were counted by Cherenkov counting in a Tricarb liquid scintillation counter (Packard Ltd.) with an open channel. A zero time point was spotted on to a filter paper square before the addition of enzyme protein. The reaction was linear up to 5 minutes.

iv. Incubation with cAMP-dependent Protien Kinase.

Freshly perfused rat heart homogenate, supernatant, or mitochondrial preparation (approximately 0.5mg) was incubated at 30^{o}C for 15 minutes in buffer (0.25M sucrose, 10mM Tris, 10mM magnesium chloride, 1mM EDTA pH 7.6) with, when included, 5mM ATP, 5µM cAMP, and 0.1mg cAMP-dependent protein kinase (prepared as described previously) in a final volume of 200µl. Samples were then assayed immediately after incubation for their activity of GPAT (homogenate),or TGL

(supernatant).

v. Incubation with Dephosphorylation Conditions.

The method used here is based on the method of Severson et al (1977). It depends on the activation by Ca^{2+} and Mg^{2+} of endogenous phosphoprotein phosphatase. To 500µl of whole homogenate or supernatant enzyme preparation, 50µl of 100mM $MgCl_2$, 50µl of 120mM EGTA, 50µl of 120mM $CaCl_2$, 100µl of 100mM MOPS (pH 7) were added. The mixtures were vortexed and incubated at 30°C for 30 minutes. This was then assayed for TGL and GPAT activity.

Control mixtures contained the same reagents except $MgCl_2$ and $CaCl_2$ (200µl of water was added instead). This preparation was then assayed for TGL and GPAT activity after incubation at 30°C for 30 minutes.

Section 2.5

i. Determination of Protein Content

This was based on the method of Bradford (1976) using bovine serum albumin as standard. 50mg of Coomassie blue G 250 was dissolved in 25ml of ethanol (95%). To this was added 50ml of orthophosphoric acid and made up to a final volume of 500ml with double-distilled water. The

solution was filtered twice through Whatman filter paper (No.1) before use. 2ml of this solution was added to 100µl of the test protein and the absorbance determined at 595 nm.

Section 2.6

Numerical Analysis of Results

Statistical analysis was performed according to the usual formula for Student's t-test. Differences between groups were tested for significance with an unpaired t-test. All values are presented as mean ± standard error of the mean (SEM).

Calculation of Results

i. Activity of Glycerol 3-phosphate acyltransferase (GPAT).

GPAT activity was expressed as nmole/min/mg protein.

$$\frac{\text{sample count}}{\text{standard count}} \times \text{nmoles in standard} \times \frac{1}{\text{time}} \times \frac{1000}{\mu\text{g protein in aliquot}} \times \frac{300}{100}$$

The standard count was measured by taking 10µl from the assay mixture (prepared as described) added to 2ml of scintillation fluid, vortexed and counted.

ii. Activity of Triglyceride Lipase (TGL)

TGL activity was expressed as nmole/min/mg protein. To calculate the standard count, 10µl from the assay mixture (prepared as described earlier) was added to 4ml of scintillation fluid, vortexed and counted.

$$\frac{\text{sample count}}{\text{standard count}} \times \text{nmoles in standard} \times \frac{1}{\text{time}} \times \frac{1000}{\mu\text{g protein in aliquot}} \times \frac{210}{50}$$

iii. Activity of Carnitine Palmitoyl Transferase (CPT)

CPT activity was expressed as nmole/min/mg protein. To calculate the standard count 10µl of the assay mixture was added to 2ml of scintillation fluid, vortexed and counted.

$$\frac{\text{sample count}}{\text{standard count}} \times \text{nmoles in standard} \times \frac{1}{\text{time}} \times \frac{1000}{\mu\text{g protein in aliquot}} \times \frac{300}{100}$$

Section 2.7

Materials

i. Perfusion Equipment

0.45µm membrane filters were obtained from Sartorius GmbH, Gottingen, West Germany (cat. no. 11306) or from Millipore, London (cat. no. HAWPOY7000). The 25mm diameter prefilters were obtained from Millipore (cat. no. AP25025000). The membrane filter housing was a Millipore Swinnex 25.

Silicone tubing for the pump and glass to polythene tubing connections were from Watson Marlow, Falmouth, Cornwall. The dimensions were id 3.2mm, od 6.4mm.

Polythene transmission tubing was supplied by Portex Ltd. (Hythe, Kent) (cat. no.800/100/280).

Perfusion glassware was obtained from Jencons Ltd. (Hemel Hempstead, Herts).

Physiological recorders (M19) were from Devices Instruments Ltd. (Welwyn Garden City, Herts).

Tricarb liquid scintillation counter was from Packard Instruments Ltd. (Caversham, Berks).

ii. Chemicals

Laboratory chemicals were Analar grade from BDH Ltd. Poole Dorset.

Sigma Chemical Co. supplied; glycerol 3-phosphate (ammonium salt), palmitoyl CoA, bovine serum albumin

(fraction V powder), streptomycin sulphate, pencillin, neomycin, adrenaline, clonidine, yohimbine, isoprenaline, atenolol, DL-carnitine hydrochloride, 6-hydroxydopamine.

Radiochemicals were from The Radiochemical Centre, Amersham, Bucks.

Methoxamine was a gift from Welcome Research, Beckenham, Kent.

Doxazosin was a gift from Pfizer Central Research, Sandwich, Kent.

RESULTS

Section 3.1

Characteristics of Enzyme Assays

i. Triglyceride Lipase (TGL) Activity.

The TGL activity of the isolated perfused rat heart was measured by the method of Severson (1979) (see methods section). The differentiation between TGL and lipoprotein lipase activity has been extensively characterised in previous work in this laboratory (Al-Muhtaseb, 1982). The experiments described in this section and in previous work (Al-Muhtseb, 1982) provide the basis for the incubation conditions adopted for subsequent routine assays.

a) Effect of Incubation Time

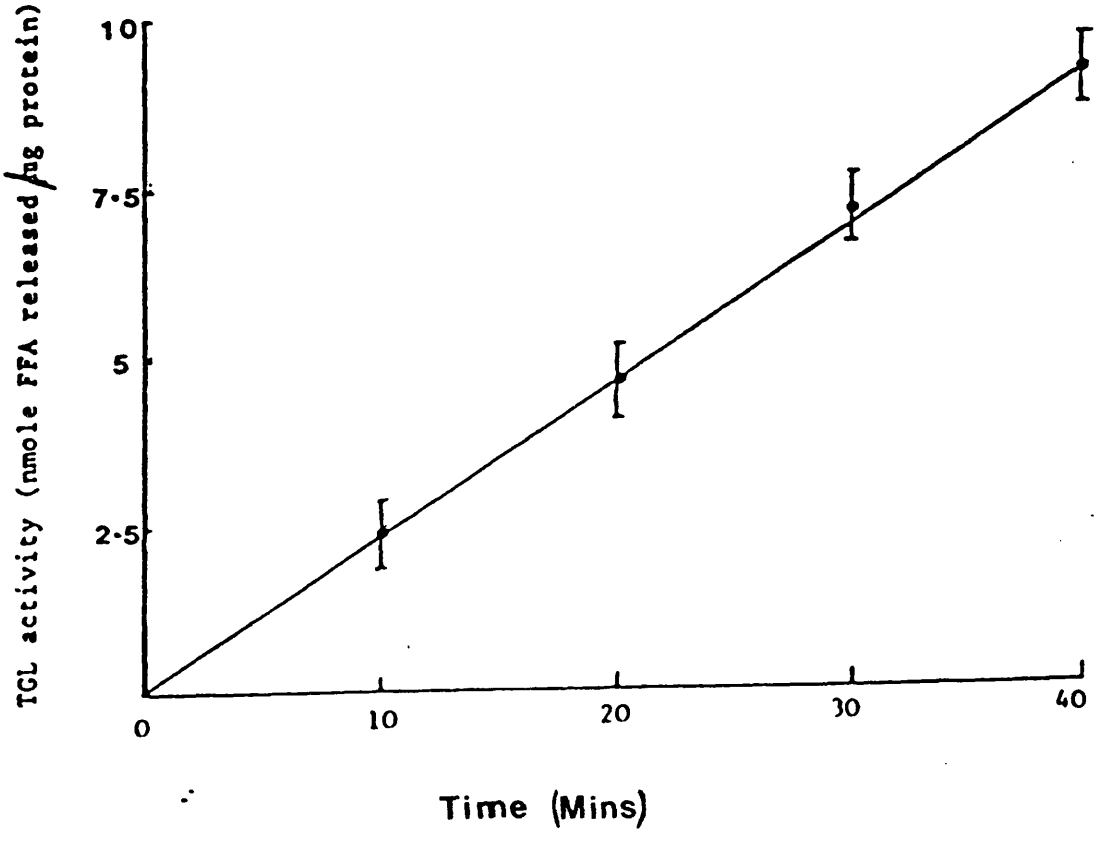
The effect of incubation time (0,20,30 and 40 minutes) on the activity of TGL measured in rat heart supernatant was studied. The incubation medium and homogenisation procedure were as descibed in the methods section. It can be seen from fig 3.1 that the rate of free fatty acid (FFA) release was linear over the 40 minutes. A similar result was observed by Severson (1979) for rat heart fractions and Vaughan et al (1964) for rat adipose tissue. An incubation time of 30 minutes was adopted as routine.

Figure 3·1

Effect of Incubation Time Against TGL Activity.

No. of Measurements = 6

Values are Means ± S.E.M.



b) Effect of Enzyme Concentration

The relationship of TGL activity to the concentration of supernatant added to the assay is shown in fig 3.2.

The assay conditions were identical to that detailed in the methods section except that different concentrations of supernatant protein were added to the assay. TGL activity is linear with increasing amounts of protein up to at least 100µg of protein added per 80µl of assay. Consequently, all subsequent determinations were performed using between 20 and 40µg of protein per 80 µl of assay.

c) Other Characteristics

The effects of triolein concentration, homogenisation time and pH on TGL activity are reported in a previous work from this laboratory (Al-Muhtaseb, 1982).

ii. Glycerol 3-phosphate Acyltransferase (GPAT) Activity

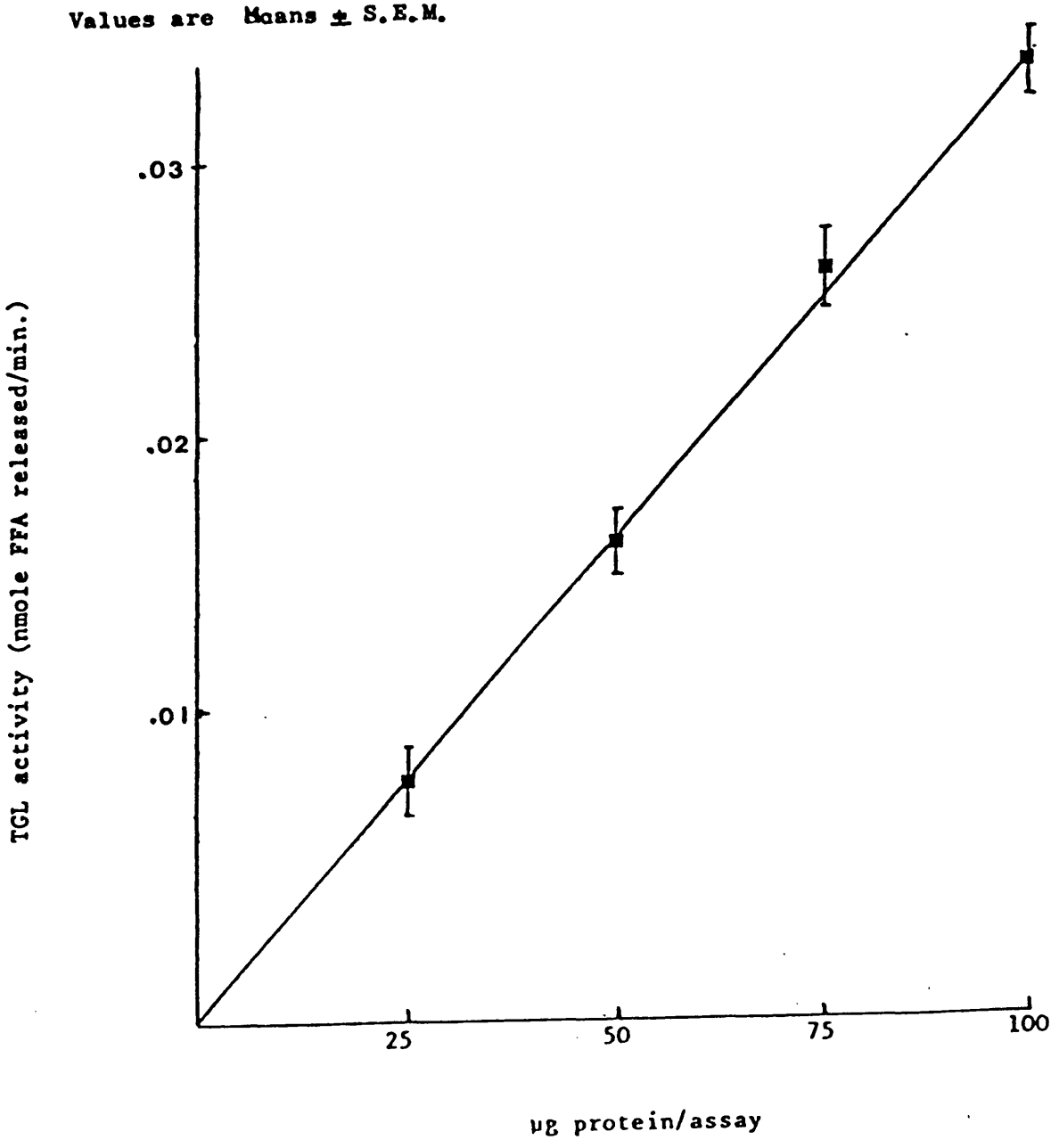
GPAT activity was measured by a direct method using glycerol 3-phosphate and palmitoyl CoA (Brands et al, 1963 and Bates and Saggerson, 1977). Other workers have used different methods. One using palmitate, CoA and ATP to

Figure 3·2

Effect of Enzyme Concentration Against TGL Activity.

No. of Observations = 6

Values are Means ± S.E.M.



generate palmitoyl CoA assumes that palmitoyl CoA synthetase is not rate-limitting (Angel and Roncari, 1967; Lamb and Fallon, 1970; Breach and Dils, 1975 and Jamdar et al, 1978). Alternatively, some groups have used palmitoyl carnitine, CoA and added the enzyme carnitine palmitoyl transferase to obtain palmitoyl CoA. This gave comparable rates of esterification to that seen with palmitate or palmitoyl CoA (Daae and Bremer, 1970 and Nimmo, 1979).

In this study the direct method was used based on the method described by Evans (1977). This assay method has the advantage of not relying on another enzyme system which could, under abnormal conditions, be rate-limiting.

a) The Effect of Incubation Time

The effect of incubation time on GPAT activity can be seen in fig 3.3. It can be seen that esterification of glycerol 3-phosphate was linear up to 5 minutes. Beyond this time linearity was never achieved in agreement with Evans (1977) and Jamdar and Fallon (1973) using adipose tissue homogenates. Possible reasons for the decrease in the rate are exhaustion of one or more of the substrates, or inactivation of the enzyme by a factor produced during incubation (end product inhibition). However, no satisfactory explanation for this phenomenon has been reported. Therefore, the standard assay is incubated for 5 minutes and linearity checked by measurements at 2.5 minutes.

b) The Effect of Enzyme Concentration

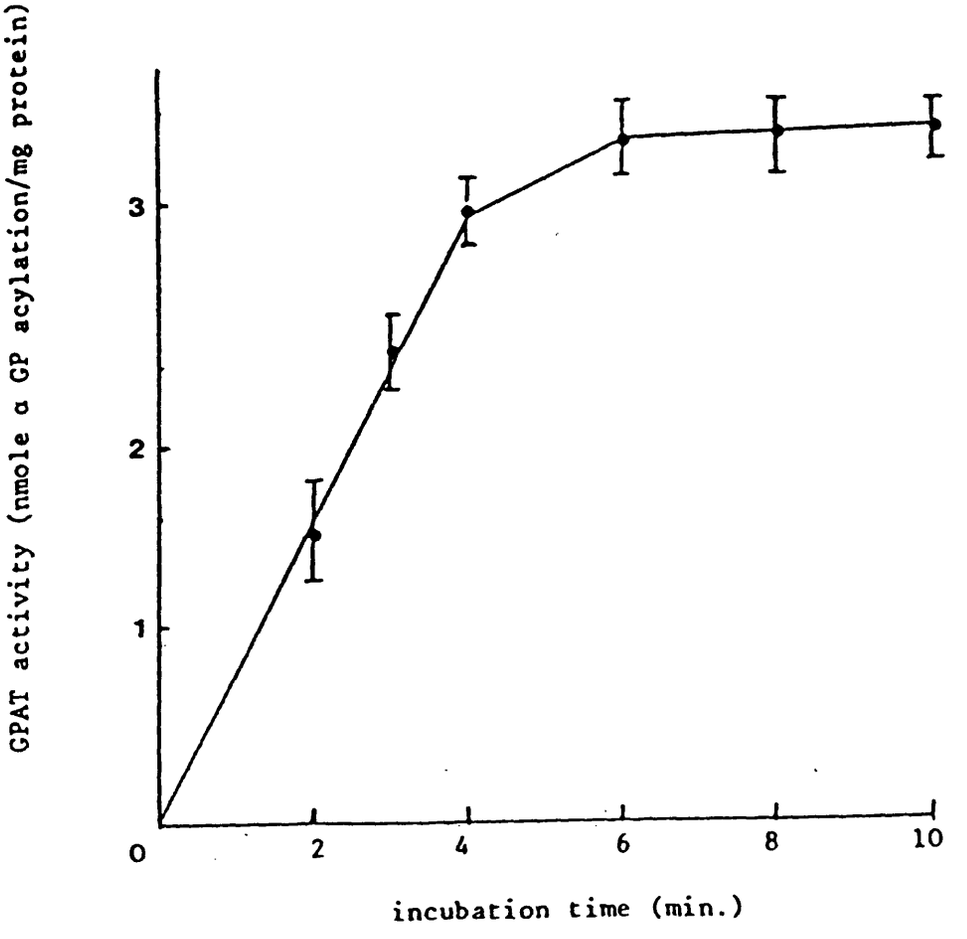
The relationship of activity to the concentration

Figure 3·3

Effect of Incubation Time Against GPAT Activity.

No. Of Observations = 6

Values are Means ± S.E.M.



of homogenate assayed is shown in fig 3.4. The assay conditions were identical with those detailed in the methods section except that different homogenate concentrations were added to the assay.

It can be seen that GPAT activity is linear with increasing amounts of protein up to at least 130 µg of protein per 100µl of assay. All subsequent determinations were performed with homogenate additions of between 50 and 70 µg of protein.

c) Other Characteristics

Previous work in this laboratory has detailed the effects of different concentrations of palmitoyl CoA, glycerol 3-phosphate and albumin. The final assay system is detailed in the methods section using 100µm palmitoyl CoA, 0.5mM glycerol 3-phosphate and albumin (2 mg/ml).

d) Identification of Lipid End Products of Glycerol 3-Phosphate Acylation

Previous studies have suggested that the major end products of the glycerol 3-phosphate esterification system in vitro were mainly phosphatidate and to a lesser extent lysophosphatidate (Lands and Hart, 1965 and Evans, 1977).

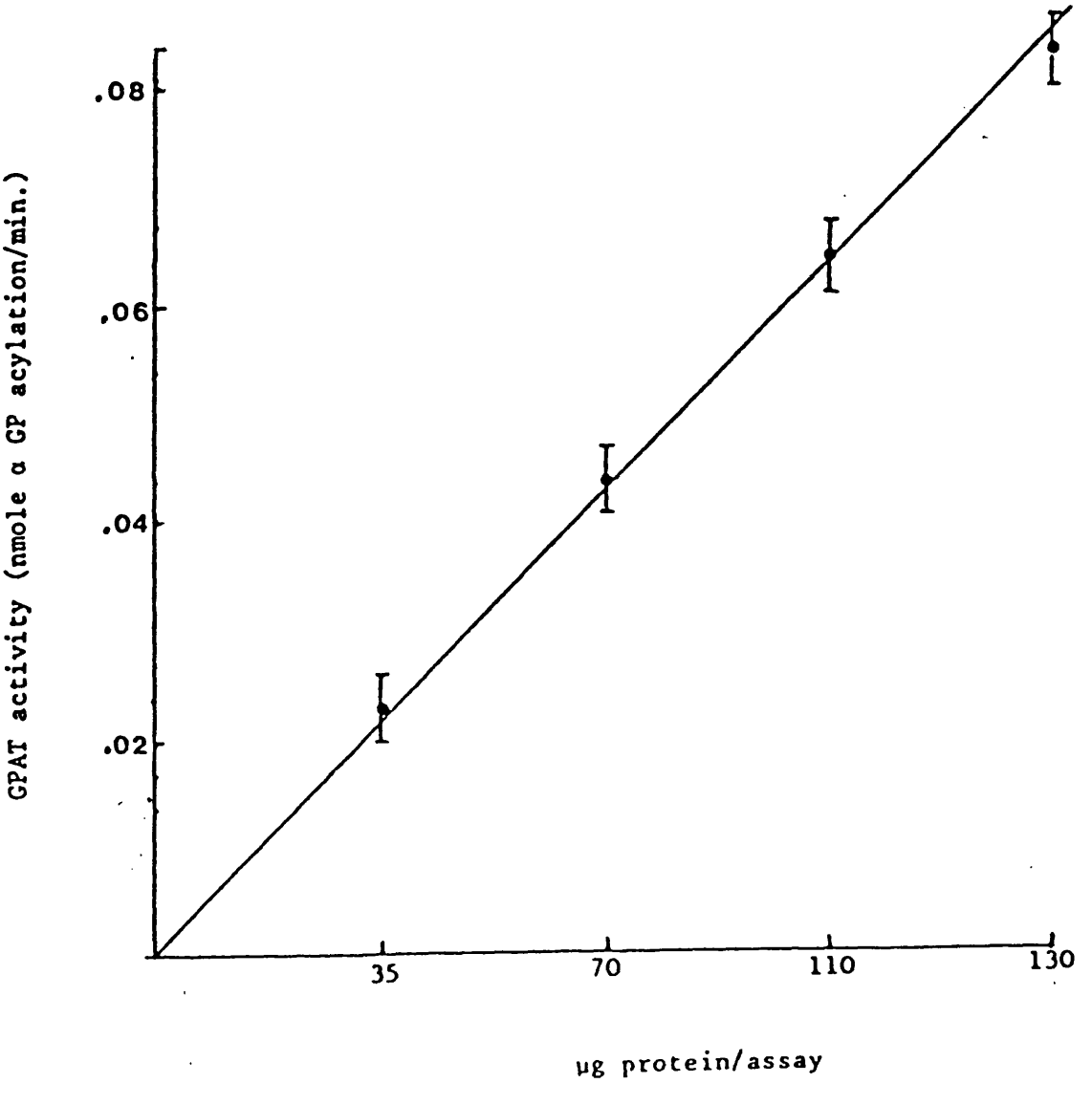
In previous work in this laboratory the end-products of acylation by perfused rat heart homogenate was examined by thin-layer chromatography. It was found that the majority (79%) of the end-product was phosphatidate.

Figure 3·4

Effect of Enzyme Concentration Against GPAT Activity.

No. of Observations = 6

Values are Means ± S.E.M.



Thus, the acylation system appears similar to that reported for other tissues and there is little or no accumulation of triglyceride or monoacyl glycerol 3-phosphate.

iii. Carnitine Palmitoyl Transferase (CPT) Activity

This assay was based on the method of Saggerson (1982). The effects of changing the assay parameters are described by Saggerson (1982) and McGarry et al (1983).

a) The Effect of Incubation Time

The results of measuring the formation of palmitoyl carnitine against time can be seen in fig 3.5. CPT activity is linear up to 3.5 minutes under the assay conditions described in the methods section. Subsequently CPT activity was measured over a period of 3 minutes.

b) The Effect of Different Concentrations of Mitochondrial Protein

CPT activity measured after the addtion of different amounts of mitochondrial protein is shown in fig 3.6. It can be seen that the assay is linear at least up to the addition of 40 μg of mitochondrial protein. In subsequent determinations 10 to 20 μg of mitochondrial protein was

FIG 3·5

CPT Activity Against Time

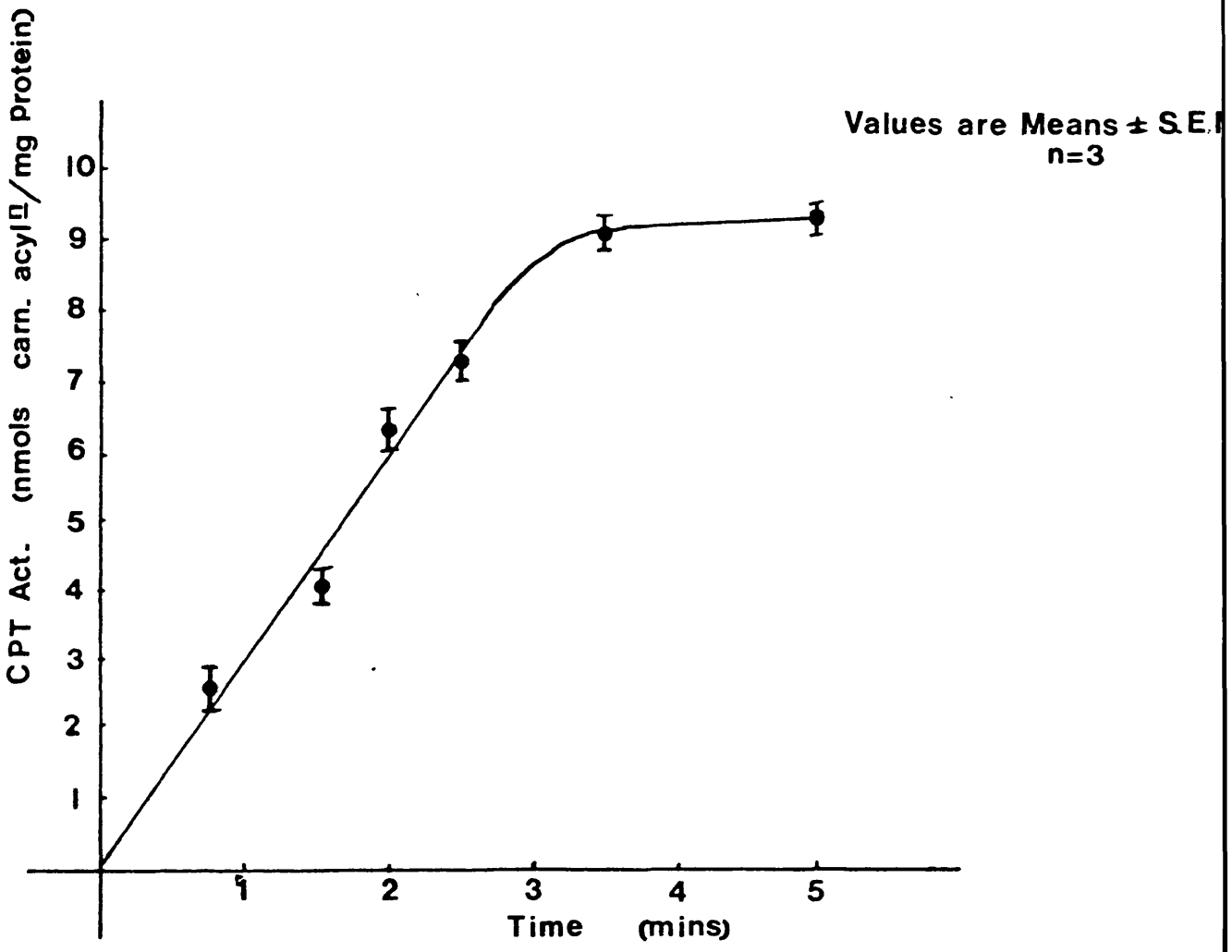
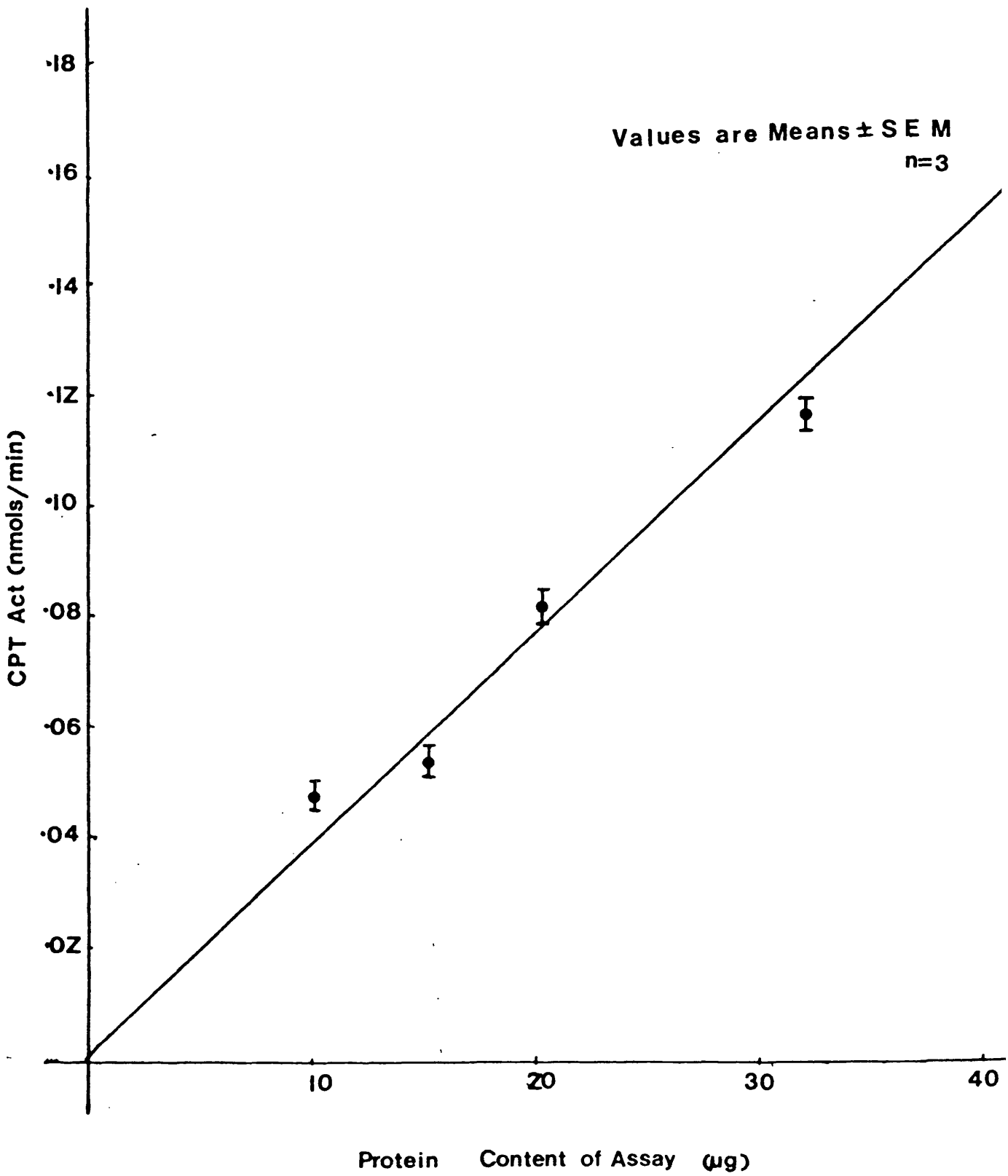


FIG 3·6

CPT Activity Against Protein Added To Assay



assayed for the activity of CPT.

Section 3.2

The Effect of Catecholamines on Lipid Metabolising Enzymes in the Normal Rat Heart.

Introduction

It has been shown that perfusion of the heart with catecholamines increases the rate of cardiac lipolysis (Gartner and Vahouny, 1973; Crass et al, 1975; Hron et al, 1977; Jesmok et al, 1977; Hulsmann and Stam, 1978 and Severson et al, 1980). This stimulation can be blocked by β-adrenergic antagonists (Christian, 1969 and Jesmok et al, 1977). The β-adrenergic effects of catecholamines on cardiac metabolism are generally considered to be mediated through increased tissue levels of cAMP and subsequent phosphorylation of key rate-limiting enzymes by cAMP-dependent protein kinase (for review see Mayer, 1974).

Bjorntorp and Furman (1962) first reported direct evidence of a stimulation of cardiac triglyceride lipase (TGL) activity by adrenaline in rat heart homogenates. However, Severson (1979) was unable to confirm this possibly due to his use of previously frozen tissue.

The inhibition of triglyceride synthesis could also lead to increased net triglyceride mobilization. Although no effects of catecholamines on the activities of triglyceride-synthesising enzymes in the heart have been reported, there is evidence to show that glycerol

3-phosphate acyltransferase (GPAT) is inhibited by catecholamines in adipose tissue (Sooranna and Saggerson, 1976,78). It has been suggested that this could be mediated by a cAMP-dependent phosphorylation system (Nimmo and Houston, 1978).

The activity of carnitine palmitoyl transferase (CPT) has been shown to regulate the activity of the β -oxidation pathway (McGarry and Foster, 1980). CPT activity has not been shown to respond to catecholamines in any mammalian tissue.

The purpose of the present study was to investigate the action of catecholamines on the activities of TGL, GPAT and CPT in the isolated-perfused rat heart. The activities of all three enzymes were measured at the same time and in the same heart. Details of the perfusion protocol and enzyme assays are given in the methods section.

i. The Effect of Adrenaline Perfusion

Adrenaline was introduced into the perfusion apparatus to reach a final concentration of 10^{-6}M. The expected increase in both the heart rate and the developed tension was seen. An average increase in heart rate was from 250 to 350 beats per minute whilst developed tension increased approximately 50% from 4g to 6g. The increased mechanical performance of the heart reached a maximum at 1.5 minutes after adrenaline addition whereupon the hearts were dismounted, homogenised and the activities of TGL, GPAT and

CPT were measured as detailed in the methods section.

Table 3.1 shows the activities of TGL, GPAT and CPT at the time of the peak adrenaline effect. It can be seen that TGL activity was increased by 50% above its control value while GPAT activity was decreased by 48% below its control value. No change was seen in CPT activity.

Ten minutes after adrenaline infusion the mechanical performance of the heart had returned to normal. Table 3.1 also shows that the activities of TGL and GPAT had returned to normal 10 minutes after adrenaline infusion.

ii. The Effect of Adrenaline on Mitochondrial and Microsomal GPAT Activity

The isoenzymes of GPAT are found in the mitochondrial and microsomal fractions of most mammalian tissues (for review see Bell and Coleman, 1980). Rider and Saggerson (1983a) found that both the mitochondrial and microsomal forms of the enzyme in rat adipocytes respond to noradrenaline. No information is available about such effects in the heart and it was decided to examine this. It is possible to distinguish between the two types by inhibiting the microsomal form with N-ethylmaleimide (NEM) (Bates and Saggerson, 1977; Monroy et al, 1972 and Haldar et al, 1979). Table 3.2 shows the effect of incubating homogenates from control or adrenaline-treated hearts with 15mM NEM for 5 minutes at 30^{o}C.

It can be seen that, in the control heart, the

Table 3.1

The effect of adrenaline, at 10^{-6}M, on the activities of triglyceride lipase (TGL), glycerol 3 -phosphate acyltransferase (GPAT) and carnitine palmitoyl transferase (CPT_I) in rat heart supernatant, homogenate and whole mitochondria.

	Enzyme Activity (nmole/min/mg protein)		
	TGL	GPAT	CPT
Control	(9)0.20±0.03	(14)0.63±0.07	(6)4.60±1.0
Adrenaline	(7)0.30±0.05**	(10)0.30±0.05***	(6)4.91±0.07
10 min. after peak adrenaline	(6)0.18±0.02	(6)0.60±0.03	(6)4.82±0.09

Values are expressed as (no. of observations) means ±S.E.M. Statistical significance compared to control values, *= $P<0.05$, **= $P<0.01$, ***= $P<0.001$.

Table 3.2

The response of NEM-sensitive (microsomal) and NEM-insensitive (mitochondrial) glycerol 3-phosphate acyltransferase (GPAT) activity to adrenaline (10^{-6}M).

	GPAT Activity (nmole/min/mg protein)	
	NEM-sens(microsomal)	NEM-insens(mitochondrial)
Control	(8) 0.35±0.04	(8) 0.23±0.03
Adrenaline	(9) 0.16±0.05**	(6) 0.14±0.02*

Values are (no. of observations) mean±S.E.M.

Statistical significance v control, *= P,0.05, **= P<0.01.

NEM-sensitive (microsomal) GPAT activity accounts for approximately 60% of the total activity. Mitochondrial (NEM-insensitive) activity accounts for 40%. Thus the proportion of GPAT activity in heart due to the mitochondrial fraction is similar to the liver but considerably greater than adipose tissue (Bates and Saggerson, 1977; Monroy et al, 1972 and Haldar et al, 1979).

Adrenaline reduced the microsomal activity by 54% and the mitochondrial activity by 39%. Thus, adrenaline appears to decrease both forms of the enzyme but particularly the microsomal form.

iii. The Effect of Adrenaline on CPT_I and CPT_{II} Activities.

Carnitine palmitoyl transferase is located on the outer (CPT_I) and inner (CPT_{II}) surfaces of the inner mitochondrial membrane (McGarry and Foster, 1980). CPT_I activity was measured with whole mitochondria. Disruption of the mitochondria by sonication revealed the total CPT activity, the difference being the latent activity or CPT_{II} activity (see Methods).

Table 3.3 shows the activities of CPT_I and CPT_{II} both before and after adrenaline infusion. No change was seen in the activity CPT_I , CPT_{II} or the total CPT activity.

iv. The Effect of Adrenaline on the Sensitivity of CPT_I

Table 3.3

The effect of adrenaline (10^{-6}M) on total CPT, CPT_I and CPT_{II} activities in rat heart mitochondria.

CPT Activity (nmole/min/mg protein).

	Total CPT	CPT_I	CPT_{II}
Control	6.80±1.21	4.60±1.0	2.41±0.07
Adrenaline	7.80±1.13	4.91±0.7	3.45±1.13

Number of observtion = 6

Values are means±S.E.M.

Table 3.4.

The effect of adrenaline on the inhibition of CPT_I by malonyl CoA.

Malonyl CoA conc (μM)	CPT_I Activity	
	Control (% inhib.)	Adrenaline (% inhib.)
0	4.82±0.44	4.88±0.30
3	3.40±0.53 (29)	3.66±0.38 (25)
15	2.55±0.50 (47)	2.83±0.39 (43)

Number of observations = 6

Values are means±S.E.M.

Activity to Malonyl CoA.

Malonyl CoA is a potent inhibitor of CPT_I but not CPT_{II} activity (McGarry and Foster, 1978). It is a physiological regulator of CPT activity and hence beta-oxidation of fatty acid in the heart where the concentration of malonyl CoA has been shown to decrease on starvation (McGarry and Foster, 1978). The sensitivity of CPT activity to malonyl CoA has been shown to vary with the nutritional (Saggerson and Carpenter, 1981) and thyroidal (Stakkestad and Bremer, 1983) state of the animal.

Table 3.4 shows the effect of two concentration of malonyl CoA on the activity of CPT_I measured in whole mitochondria prepared from control and adrenaline-treated hearts. The concentration of 3μM was chosen because it has been reported to give approximately 50% inhibition (Saggerson, 1982). If a change in the sensitivity of CPT_I to malonyl CoA were to occur it would probably be most clearly evident around this concentration. 15μM is reported to give a very high degree of inhibtion (80%) (Saggerson, 1982).

It can be seen from table 3.4 that 3 μM malonyl CoA caused 30% inhibition and 15μM caused 48% inhibition in mitochondria from control hearts. These values were not significantly altered in whole mitochondria from adrenaline-treated hearts. Thus, adrenaline did not alter the sensitivity of CPT_I to malonyl CoA.

These % inhibition values are rather low compared to the results of Saggerson (1982). This would suggest that some of the mitochondria were in a broken state and that CPT_{II} was also assayed. As CPT_{II} is not inhibited by malonyl

CoA (McGarry et al, 1978b), this fraction will remain active when the concentration of malonyl CoA is large enough to completely inhibit CPT_I.

Section 3.3

The Effect on enzyme activites of Incubation with cAMP-dependent Protein Kinase and Phosphatase Conditions.

In rat adipose tissue, adrenaline has been shown to activate TGL via a cAMP-mediated, reversible phosphorylation system ("the lipolytic activation cascade") (Steinberg and Huttunen, 1970 and Stralfors and Belfrage, 1983). GPAT activity is reduced when adipocytes are exposed to adrenaline (Sooranna and Saggerson, 1976), possibly by a similar cAMP-mediated phosphorylation mechanism (Nimmo and Houston, 1978). If the adrenaline effects reported in the previous section are similar to those seen in adipose tissue it might be expected that cAMP-dependent protein kinase would have an effect on these enzymes.

Homogenates and 2,000g supernatants from hearts perfused in the absence of adrenaline for 20 minutes were incubated for 15 minutes with cAMP-dependent protein kinase, together with cAMP and ATP (see Methods section). The activities of TGL (supernatant) and GPAT (homogenate) were then measured. The results are shown in Table 3.5.

With cAMP-dependent protein kinase, cAMP and ATP present TGL activity increased to 0.22±0.04 above a control value of 0.17±0.03. GPAT activity was reduced under the same conditions from 0.58±0.03 to 0.28±0.03. No changes were seen in the absence of added protein kinase, of ATP, or in the absence of cAMP.

TABLE 3.5

The effect of cAMP-dependent Protein Kinase on the activity of Triglyceride lipase (TGL) and Glycerol 3-phosphate acyltransferase (GPAT) in rat heart supernatant or homogenate.

INCUBATION	ENZYME ACTIVITY (nmole/min/mg protein)	
	TGL	GPAT
Buffer alone	0.17±0.03	0.59±0.01
Buffer + ATP	0.17±0.04	0.59±0.05
Buffer + cAMP + ATP	0.15±0.01	0.58±0.03
Buffer + cAMP + cAMP-depend. Protein Kinase	0.16±0.02	0.58±0.03
cAMP + ATP + cAMP-depend. Protein Kinase	0.22±0.04*	0.28±0.03**

Number of experiments = 3 (separate perfused hearts).
Values are means±S.E.M.
Statistical significance v Control, * = $P < 0.05$, ** = $P < 0.01$.
Incubations for 15 minutes at 30°C as detailed in the methods section.

The catalytic subunit of cAMP-dependent protein kinase was prepared as detailed in the methods section. This was used to study the effect of cAMP-dependent phosphorylation on mitochondrial and microsomal forms of GPAT. Fractionation of the homogenate into mitochondrial and microsomal (10,000g supernatant) samples was by centrifugation as described in the methods section.

As can be seen in fig 3.7 that the catalytic subunit decreased microsomal GPAT activity in a time-dependent manner but did not alter mitochondrial activity. Thus, the mitochondrial activity must respond to adrenaline by a different mechanism, possibly by a cAMP-independent phosphorylation system.

Similarly, incubation of the 2,000g supernatant with the catalytic subunit resulted in a slight increase in TGL activity over 20 minutes whereas the control value fell (fig 3.8).

When using tissue from hearts perfused with adrenaline (Table 3.6) no activation of TGL activity by cAMP-dependent protein kinase above the already activated level was seen. Similarly, GPAT activity was not changed from the low level seen in tissue from adrenaline-treated hearts by incubation under phosphorylation conditions.

Table 3.6 also shows the results of incubation of tissue from adrenaline-treated hearts with the dephosphorylation conditions detailed in the methods section. This method relies on the activation of endogenous phosphatase by Ca^{2+} and Mg^{2+} ions (Severson et al, 1977). After 30 minutes incubation under dephosphorylation conditions the activated TGL activity had fallen to the

Incubation with the Catalytic Subunit of cAMP dependent Protein Kinase

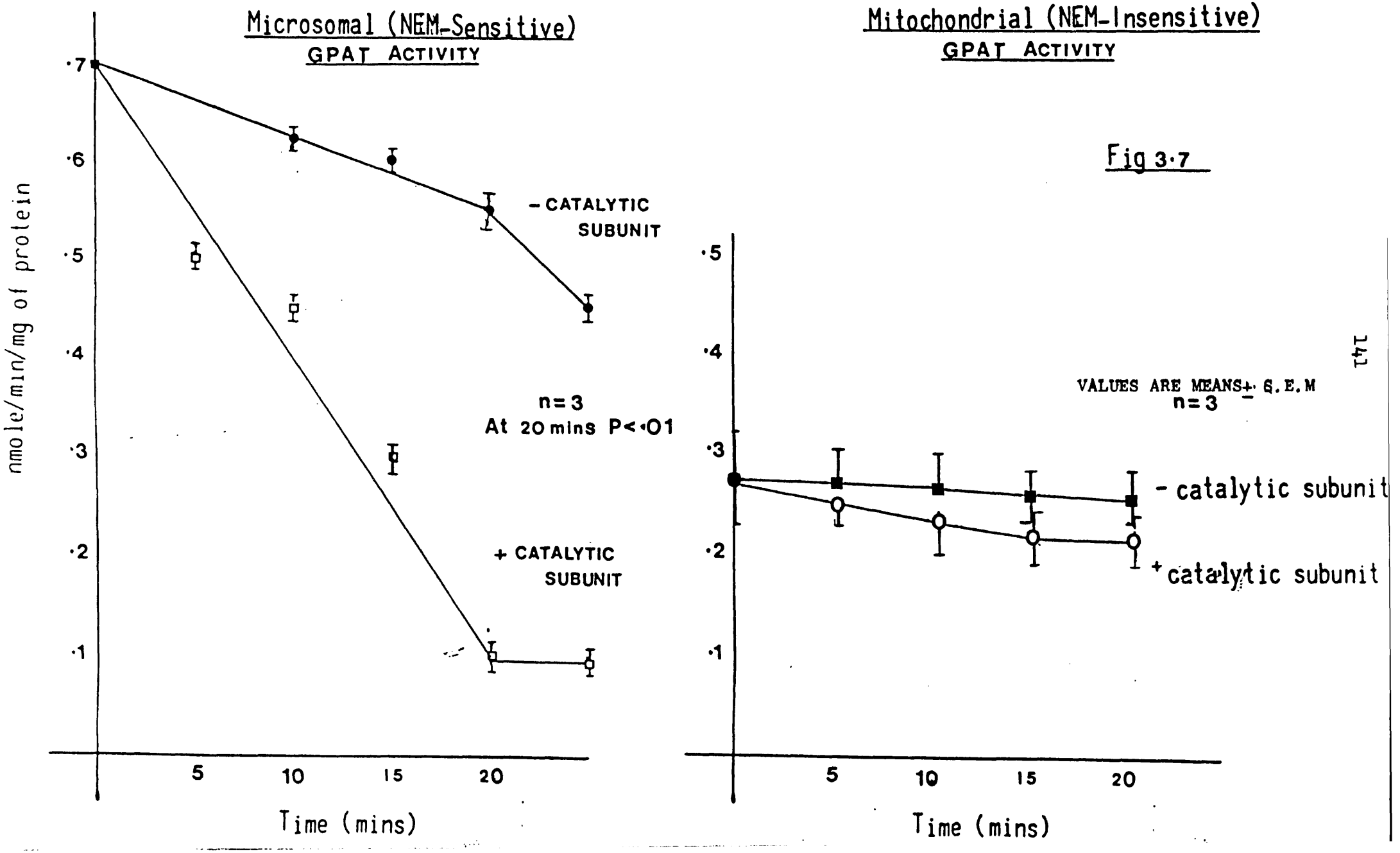


Fig 3·7

FIG 3·8

Incubation with the Catalytic Subunit of cAMPdependent Protein Kinase

TGL ACTIVITY

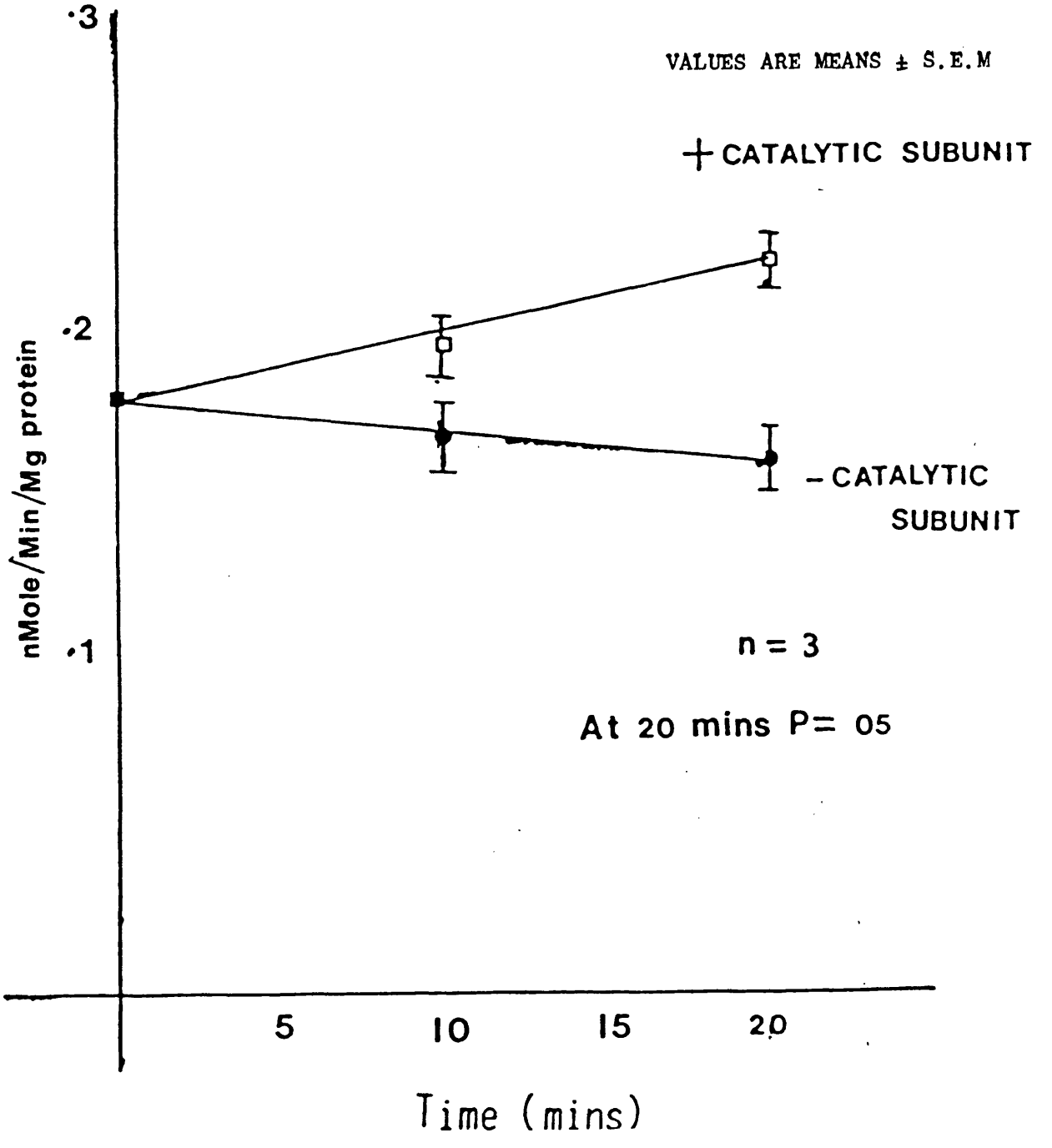


TABLE 3.6

The effect of cAMP-dependent Protein Kinase and Dephosphorylation Conditions on the Activity of Glycerol 3-phosphate acyltransferase (GPAT) and Triglyceride lipase (TGL) in Rat Hearts Perfused with Adrenaline.

INCUBATION	ENZYME ACTIVITY (nmole/min/mg protein)	
	TGL	GPAT
Phosphorylation controls (Table 2)	0.35±0.04	0.30±0.02
cAMP + ATP + cAMP-depend. Protein Kinase	0.37±0.03	0.29±0.02
Dephosphorylation control (without Ca^{2+},Mg^{2+})	0.31±0.04	0.32±0.04
Dephosphorylation Conditions (with Ca2+,Mg2+)	0.19±0.03*	0.55±0.07*

Number of experiments = 3 (separate adrenaline perfused hearts).

Values are means±S.E.M.

Statistical significance v Control, * = $P< 0.05$.

Incubations for 15 minutes (phosphorylation) or 30 minutes (dephosphorylation) at 30°C as detailed in the methods section.

activity measured in control hearts. Similarly, the inactivated GPAT activity had risen to control values. Thus the activation of TGL and inactivation of GPAT can be reversed under dephosphorylation conditions.

These results are consistant with those found for the same enzyme activities in adipose tissue and suggest that at least some of the effects produced by adrenaline could be mediated by cAMP-dependent protein kinase phosphorylations.

Section 3.4

The Effect on the Enzyme Activities of Perfusion with Adrenergic Agonists and Antagonists

i. Perfusion with Alpha and Beta Adrenergic Agonists

Introduction

Adrenaline has been shown to activate both α and β adrenergic mechanisms (Lands et al, 1967 and Berthelson and Pettinger, 1977). Both these systems are present in the rat myocardium (Benfey, 1973; Alexander et al, 1975; Hancock et al, 1979 and Williams and Lefkowitz, 1978). Table 3.7 shows the effect on TGL, GPAT and CPT_I activities of perfusing the isolated rat heart with either the α adrenergic agonist phenylephrine or the β adrenergic agonist isoprenaline at a concentration of 10^{-6}M.

It can be seen that phenylephrine induced a fall in TGL activity and a rise in GPAT activity. This is in accord with the fall in cAMP levels produced by phenylephrine in the isolated rat heart (Keely et al, 1977) and in rat myocytes (Watanabe et al, 1977).

No change was seen in the activity of CPT.

The changes in enzyme activity produced by the β adrenergic agonist isoprenaline were in the same direction as those produced by adrenaline infusion but of a greater magnitude. This is consistant with the greater increase in cAMP levels produced by isoprenaline than by adrenaline in rat adipose tissue (Fain, 1973).

TABLE 3.7

The effect of perfusing the heart with phenylephrine (alpha-adrenergic agonist) and isoprenaline (beta-adrenergic agonist) at 10^{-6}M on the activities of TGL, GPAT and CPT_I.

	Enzyme Activities (nmoles/min/mg protein)		
	TGL	GPAT	CPT_I
Control	(8) 0.22±0.05	(14) 0.63±0.07	(10) 5.87±0.92
Phenylephrine	(7) 0.15±0.02**	(6) 0.75±0.06**	(6) 5.65±1.29
Isoprenaline	(7) 0.42±0.07**	(9) 0.31±0.05***	(6) 5.38±1.67

Values are (number of observations) means ± S.E.M.

Statistical siginificance v control;

*= P< 0.05, **= P< 0.01, ***= P< 0.001.

Table 3.8.

The effect of infusion of the alpha-adrenergic agonist Phenylephrine (10^{-6}M) and the beta-adrenergic agonist Isoprenaline (10^{-6}M) on the activity of microsomal (NEM-sensitive) and mitochondrial (NEM-insensitive) GPAT.

	Enzyme Activity (nmole/min/mg protein)	
	Microsomal (NEM-sens)	Mitochondrial (NEM-insens)
Control	(8) 0.35±0.04	(8) 0.23±0.03
Phenylephrine	(6) 0.46±0.05*	(6) 0.20±0.05
Isoprenaline	(5) 0.17±0.08*	(5) 0.19±0.03

Values are (number of observations) means ± S.E.M.

Statistical significance v control; *= P< 0.01.

Thus, the changes in enzyme activities produced by α and β adrenergic stimulation appear to be in opposite directions.

Table 3.8 shows the effect of phenylephrine and isoprenaline infusion on the activities of mitochondrial and microsomal GPAT. It appears that the changes in GPAT activity seen with both the α and β adrenergic agonists are localised mainly in the microsomal fraction.

ii. Perfusion with Alpha $_1$ and Alpha $_2$ Adrenergic Agonists.

Introduction

The α-adrenergic system has been divided into α_1 and α_2 on the basis of the ability of different agonists and antagonists to stimulate or inhibit the α receptor (Wikberg, 1979). It has been shown that stimulation of α_1 and α_2 receptors results in different intracellular signals (Wikberg, 1979). α_2 -adrenergic stimulation involves a reduction of cAMP levels (Wright and Simpson, 1981 and Burns et al, 1981), while the main intracellular signal generated by stimulation of α_1 receptors appears to be the increase of cytosolic free Ca^{2+} (Michell and Kirk, 1981).

Stimulation of α_2 receptors has also been shown to reduce endogenous noradrenaline release via pre-synaptic α_2 receptors (Dart et al, 1984a; Langer, 1974 and Starke et al, 1971a,b).

Both α_1 and α_2 receptors have been shown to exist in the rat heart (Benfey, 1973 and Williams and

Lefkowitz, 1978). The effects of perfusing the isolated rat heart with the α agonists clonidine (α_2) and methoxamine (α_1) on the activities of TGL and GPAT are shown in Table 3.9.

It can be seen that clonidine infusion at a concentration of 10^{-5}M resulted in a fall in TGL activity and a rise in GPAT activity. This result is similar to the α_2 adrenergic inhibition of lipolysis observed in other mammalian tissues (Lafontan and Berlan, 1980) and is consistant with a fall in cAMP levels.

The concentration of 10^{-5}M was used as this appeared maximal. At 10^{-6}M the changes in TGL and GPAT activities were similar to those reported but not as great.

Infusion of the α_1 adrenergic agonist, methoxamine, at a concentration of 10^{-6}M, did not change TGL activity (Table 3.9). However, a marked reduction was seen in GPAT activity. Thus, it appears that α_1 adrenergic mechanisms could mediate a reduction in GPAT activity.

Table 3.10 shows the effects of clonidine and methoxamine infusion on the mitochondrial and microsomal GPAT activities. It can be seen that the changes seen in total GPAT activity are localised mainly in the microsomal or NEM-sensitive fraction. A tendency for the mitochondrial activities to be stimulated by methoxamine does not reach a statistically significant level.

iii. Perfusion With Adrenergic Antagonists

Table 3.11 shows the results of continuous perfusion (10 minutes) with the cardioselective β_1

Table 3.9

The effect of infusion of the $alpha_1$ agonist Methoxamine (10^{-6}M) and the $alpha_2$ agonist clonidine (10^{-5}M) on the activities of TGL and GPAT.

	Enzyme Activity (nmole/min/mg protein).	
	TGL	GPAT
Control	(8) 0.22±0.02	(8) 0.63±0.04
Methoxamine	(6) 0.20±0.03	(8) 0.49±0.04**
Clonidine	(6) 0.18±0.01*	(6) 0.79±0.05**

Values are (number of observations) mean±S.E.M.

Statistical significance v controls; *= P= 0.05, **= P< 0.01.

Table 3.10

The effect of infusion of the alpha$_1$ agonist Methoxamine (10^{-6}M) and the alpha$_2$ agonist Clonidine (10^{-5}M) on the activity of microsomal (NEM-sensitive) and mitochondrial (NEM-insensitive) GPAT.

	Enzyme Activity (nmole/min/mg protein)	
	Microsomal (NEM-sens)	Mitochondrial (NEM-insens)
Control	(8) 0.35±0.04	(8) 0.23±0.03
Methoxamine	(5) 0.27±0.03*	(6) 0.21±0.04
Clonidine	(6) 0.50±0.08**	(5) 0.28±0.03

Values are (number of observations) mean ± S.E.M.

Statistical significance v controls; *= P< 0.05, **= P< 0.01.

Table 3.11.

The effect of continuous perfusion with atenolol (a beta-adrenergic antagonist), yohimbine (an $alpha_2$-adrenergic antagonist) and doxazosin (an $alpha_1$-adrenergic antagonist) on the activities of TGL and GPAT.

	Enzyme Activity (nmole/min/mg protein)	
	TGL	GPAT
Control	0.22±0.04	0.62±0.05
Atenolol	0.13±0.01***	0.38±0.08**
Yohimbine	0.21±0.03	0.61±0.07
Doxazosin	0.20±0.02	0.61±0.06

Number of observations = 6.

Values are means ± S.E.M.

Statistical significance v controls; *= P< 0.05, **= P< 0.01, ***= P< 0.001.

adrenergic antagonist atenolol, the α_1 adrenergic antagonist doxazosin and the α_2 adrenergic antagonist yohimbine.

It can be seen that continuous perfusion with doxazosin and yohimbine did not result in any changes in the activities of TGL or GPAT.

However, perfusion with the β_1 antagonist atenolol resulted in a fall in both TGL and GPAT activities.It appears that control hearts were still showing a degree of β -adrenergic stimulation and that perfusion with the β antagonist blocked this small adrenergic drive and reduced TGL activity. Comparison of the activity of TGL in Table 3.11 with the activity shown in Table 3.7 shows an increase of almost 4 fold from a level of 0.13 ± 0.01 under β antagonism to 0.42 ± 0.07 with β -adrenergic stimulation.

However, the fall in GPAT activity can not be explained by this mechanism as a reduced adrenergic drive would be expected to increase GPAT activity. Thus the change in GPAT activity caused by perfusion with atenolol suggests that the reduction in the activity of this enzyme seen with adrenaline may not be solely due to cAMP-dependent protein kinase effects mediated by the β -adrenergic system.

-OR ATENOLOL HAS SOME OTHER ACTION

Table 3.12 shows that the reduction in GPAT activity associated with continuous perfusion of atenolol is localised mainly in the microsomal fraction although a statistically insignificant decrease was seen in the mitochondrial fraction.

No change was seen in GPAT activity in either the microsomal or mitochondrial fractions, when yohimbine or doxazosin was present (Table 3.12).

Table 3.12.

The effect of continuous perfusion with atenolol (a β-adrenergic antagonist), yohimbine (an α_2-adrenergic antagonist) and doxazosin (an α_1-adrenergic antagonist) on the activity of the microsomal (NEM-sensitive) and mitochondrial (NEM-insensitive) forms of GPAT.

	GPAT Activity (nmole/min/mg protein)	
	Microsomal (NEM-sens.)	Mitochondrial (NEM-insens.)
Control	(8) 0.35±0.04	(8) 0.23±0.03
Atenolol	(5) 0.20±0.04*	(6) 0.16±0.03
Yohimbine	(5) 0.42±0.04	(5) 0.25±0.05
Doxazosin	(5) 0.36±0.06	(6) 0.22±0.02

Values are (number of observations) means ± S.E.M.

Statistical significance v control; *= $P < 0.05$.

Section 3.5

The Effect of Acute Ischaemia and Reperfusion on the Activities of TGL, GPAT and CPT.

Introduction

Several investigations have indicated that an increased adrenergic drive occurs in the acutely ischaemic myocardium (Abrahamsson et al, 1981; Corr et al, 1978; Holmgren et al, 1981; Podzuwiet et al, 1978; Shahab et al, 1969 and Shahab and Wollenberger, 1967). This increase in adrenergic activity may play a significant role in the development of tissue damage in ischaemia. It is believed to contribute to the occurance of ventricular arrhythmias and, by increasing metabolic disturbances, it causes myocardial cell damage (Corr and Gillis, 1978; Hjalmarso, 1980 and Maroko et al, 1971). Indeed, several authors have shown that depletion of myocardial catecholamines (Gaudel et al, 1979; Gercken and Doring, 1973 and Sakai and Spieckermann, 1975) or β-adrenergic antagonism (Sakai and Speickermann, 1975; Peiper et al, 1980; Manning et al, 1980 and Nayler et al, 1980) have been beneficial in protecting against hypoxic or ischaemically-induced injury.

One of the possible causes of cell damage and consequent myocardial dysfunction under these circumstances is the cellular accumulation of long chain fatty acids and their metabolites resulting from catecholamine-stimulated

Table 3.13.

The effect of 10 minutes ischaemia and subsequent reperfusion for 1 and 5 minutes on the activity of Triglyceride Lipase (TGL) measured in tissue from the non-ischaemic (NI) and ischaemic (I) areas of the heart.

	TGL Activity (nmole/min/mg protein)	
After 20 min.		
normal perfusion	0.18±0.02	0.18±0.02
	NI	I
After 10 min.		
ischaemia	0.18±0.03	0.31±0.04**
After isch.+ 1		
min. reperfusion	0.18±0.01	0.26±0.01***
After isch.+ 5		
min. reperfusion	0.18±0.04	0.17±0.04

Number of observations = 6

Values are means ± S.E.M.

Statistical significance v non-ischaemic area; *= P< 0.05, **= P< 0.01, ***= P< 0.001.

lipolysis of endogenous triglyceride (Opie, 1976).

In this section, the effect of acute ischaemia and subsequent reperfusion on the activities of TGL, GPAT and CPT is reported. Acute ischaemia is produced by ligation of the left coronary artery for 10 minutes as detailed in the methods section. Reperfusion of the ischaemic area, for 1 or 5 minutes, is brought about by release of the occlusion. The enzyme activities are measured in both the non-ischaemic and the ischaemic areas of the heart.

In preliminary experiments the activities of TGL, GPAT and CPT were measured in right and left ventriclular tissue (non-ischaemic and ischaemic tissue in subsequent experiments) from hearts that had undergone sham operations. It can be seen from Tables 3.13, 3.14 and 3.16 that all three enzymes showed similar activities in the two areas.

i. The Effect of Acute Ischaemia and Reperfusion on TGL Activity.

Table 3.13 shows the activity of TGL in tissue from the non-ischaemic and ischaemic areas of the heart after 10 minutes occlusion, and after 1 and 5 minutes reperfusion. In the non-ischaemic area TGL activity remained unchanged throughout occlusion and reperfusion. However, in the ischaemic area TGL activity increased by 72% after 10 minutes ischaemia. During reperfusion this high value fell to control values.

ii. The Effect of Acute Ischaemia and Reperfusion on GPAT Activity.

Table 3.14 shows the activity of GPAT in tissue from the non-ischaemic and ischaemic areas after 10 minutes occlusion and after 1 and 5 minutes reperfusion. GPAT activity remained unchanged in the non-ischaemic area throughout the occlusion and reperfusion. In the ischaemic area, GPAT activity was reduced by 28% compared to control values after 10 minutes ischaemia. After 1 minute reperfusion the activity measured in the previously ischaemic area was found to be significantly lower than the activity found after 10 minutes ischaemia ($p<0.05$). Even after 5 minutes reperfusion GPAT activity was still significantly lower than the activity seen in the non-ischaemic area or the control value. Thus, it appears that reperfusion of the ischaemic area caused a further fall in GPAT activity, below the fall seen during ischaemia.

iii. The effect of ischaemia and reperfusion on the activity of GPAT in the mitochondrial (NEM-insensitive) and microsomal (NEM-sensitive) fractions.

It can be seen from Table 3.15 that GPAT activity in both fractions remained unaltered in the non-ischaemic area throughout occlusion and reperfusion. In the ischaemic area after 10 minutes occlusion both fractions showed a lower activity than the non-ischaemic area. This change was more pronounced in the mitochondrial fraction.

After 1 minute reperfusion, microsomal activity in

Table 3.14.

The effect of 10 minutes ischaemia and subsequent reperfusion for 1 and 5 minutes on the activity of Glycerol 3-phosphate acyltransferase (GPAT) measured in tissue from the non-ischaemic (NI) and ischaemic (I) areas of the heart.

	GPAT Activity (nmole/min/mg protein)	
After 20 min.		
normal perfusion	0.60±0.08	0.61±0.03
	NI	I
After 10 min.		
ischaemia	0.63±0.04	0.46±0.07*
After isch.+ 1		
min. reperfusion	0.68±0.06	0.31±0.01***
After isch.+ 5		
min. reperfusion	0.63±0.02	0.44±0.04**

Number of observations = 6

Values are means ± S.E.M.

Statistical significance v non-ischaemic area; *= P< 0.05, **= P< 0.01, ***= P< 0.001.

Table 3.15.

The effect of 10 minutes ischaemia and subsequent reperfusion for 1 and 5 minutes on the activity of microsomal (NEM-sensitive) and mitochondrial (NEM-insensitive) Glycerol 3-phosphate acyltransferase (GPAT) measured in tissue from the non-ischaemic (NI) and ischaemic (I) areas of the heart.

	GPAT Activity (nmole/min/mg protein)			
	Microsomal (NEM-sens)		Mitochondrial (NEM-insens)	
After 20 min.				
normal perfusion	0.40±0.06	0.41±0.06	0.22±0.05	0.22±0.03
	NI	I	NI	I
After 10 min.				
ischaemia	0.43±0.04	0.35±0.03*	0.20±0.03	0.11±0.02**
After isch.+ 1				
min. reperfusion	0.45±0.05	0.17±0.03***	0.21±0.03	0.16±0.03
After isch.+ 5				
min. reperfusion	0.43±0.02	0.23±0.03***	0.20±0.02	0.21±0.02

Number of observations = 6

Values are means ± S.E.M.

Statistical significance v non-ischaemic area; *= P< 0.05, **= P< 0.01, ***= P< 0.001.

the previously ischaemic area was considerably reduced compared to the activity seen after 10 minutes ischaemia in the same area. This reperfusion-induced fall in microsomal GPAT activity was not seen in the mitochondrial fraction.

After 5 minutes reperfusion mitochondrial activity had returned to control values. At this time microsomal activity was still depressed.

Thus, it appears that ischaemia induces a fall in both the mitochondrial and microsomal GPAT activities whereas the reperfusion-induced fall in GPAT activity is evident only in the microsomal fraction.

iv. The Effect of Acute Ischaemia on the Activity of CPT.

Tables 3.16 and 3.17 show the effect of ischaemia on the activities of total CPT, CPT_I and CPT_{II} in mitochondria from non-ischaemic and ischaemic areas of the heart after 10 minutes occlusion. It can be seen that there is no change of either CPT_I , CPT_{II} or total CPT activity in the non-ischaemic or the ischaemic areas.

Thus, ischaemia does not change the activity of CPT in the rat heart.

Table 3.16.

The effect of 10 minutes ischaemia on the activity of total CPT measured in sonicated mitochondria from the non-ischaemic (NI) and ischaemic (I) areas of the heart.

	CPT Activity (nmole/min/mg protein)	
After 20 min.		
normal perfusion	7.47±0.90	6.75±1.05
	NI	I
After 10 min.		
ischaemia	6.20±0.85	6.28±0.46

Number of observations = 6

Values are means ± S.E.M.

Table 3.17.

The effect of 10 minutes ischaemia on the activities of CPT_I and CPT_{II} measured in mitochondria from non-ischaemic (NI) and ischaemic (I) areas of the heart.

	CPT Activity (nmole/min/mg protein)			
	CPT_I		CPT_{II}	
After 20 min.				
normal perfusion	5.17±0.91	5.43±1.36	2.18±1.25	2.26±1.03
	NI	I	NI	I
After 10 min.				
ischaemia	5.48±1.18	5.44±1.45	1.75±0.96	1.14±0.39

Number of observations = 6

Values are means ± S.E.M.

Section 3.6

The Effect of 6 OH-Dopamine on the Changes in TGL and GPAT Activity Induced by Acute Ischaemia and Reperfusion

Depletion of endogenous noradrenaline by injection of 6 OH-dopamine 24 hours before acute ischaemia was induced has been shown to almost completely abolish the occurance of ventricular arrhythmias during both coronary occlusion and subsequent reperfusion (Sethi et al, 1973 and Sheridan et al, 1980). Table 3.18 shows TGL and GPAT activities in hearts from animals pre-injected with 6 OH-dopamine after 10 minutes ischaemia and then 1 and 5 minutes reperfusion.

In hearts from 6 OH-dopamine-treated animals, the changes in TGL and GPAT activities produced by ischaemia and reperfusion seen previously did not occur. It can be assumed from this that the changes induced in TGL and GPAT activities by ischaemia and reperfusion in hearts not pretreated with 6 OH-dopamine, occur via an adrenergic receptor-mediated mechanism.

Table 3.18.

The effect of 10 minutes ischaemia and subsequent reperfusion for 1 minute on the activity of Triglyceride Lipase (TGL) and Glycerol 3-phosphate acyltransferase (GPAT) measured in tissue from the non-ischaemic (NI) and ischaemic (I) areas of the heart. Hearts were taken from rats which had been pre-injected with 6 OH-dopamine 24 hours previously.

	Enzyme Activity (nmole/min/mg protein)			
	TGL		GPAT	
After 20 min.				
normal perfusion	0.17±0.03	0.17±0.02	0.56±0.08	0.56±0.07
After 10 min.	NI	I	NI	I
ischaemia	0.19±0.03	0.19±0.04	0.56±0.08	0.56±0.08
After isch.+ 1				
min. reperfusion	0.17±0.03	0.17±0.04	0.59±0.06	0.62±0.04

Number of observations = 6

Values are means ±S.E.M.

Section 3.7

The Effect of Perfusion of Adrenergic Antagonists on the Changes in Enzyme Activities produced by Ischaemia and Reperfusion.

i. Pre-perfusion with the Beta Antagonist Atenolol.

The presence of a β adrenergic antagonist has been shown to reduce the cellular damage caused by ischaemia (Sakai and Speickermann, 1975; Pieper et al, 1980 and Manning et al, 1980), possibly by inhibiting catecholamine-stimulated cardiac lipolysis (Opie, 1976).

Perfusion with the cardioselective β_1 adrenergic antagonist atenolol was begun 10 minutes before occlusion of the left coronary artery occurred and thereafter for the rest of the experiment. The activities of TGL and GPAT after ischaemia and reperfusion from hearts perfused continuously in the presence of atenolol are shown in Table 3.19.

The small changes seen in the activities of TGL and GPAT in the non-ischaemic area compared to control values did not reach statistical significance.

In the ischaemic area TGL activity did not increase after 10 minutes ischaemia as seen in the absence of atenolol (Table 3.13). Similarly, there was no change in GPAT activity in the ischaemic area after 10 minutes ischaemia. This is in contrast to the decrease in GPAT activity seen in the absence of atenolol. Thus, it appears

TABLE 3.19..

The effect of continuous perfusion with the β antagonist Atenolol on the activities of Triglyceride lipase (TGL) and Glycerol 3-phosphate acyltransferase (GPAT) produced by 10 minutes ischaemia and subsequent reperfusion. Measurements were made in tissue from both the non-ischaemic (NI) and ischaemic (I) areas of the heart. Atenolol perfusion was begun 10 mins before occlusion.

	ENZYME ACTVITY (nmole/min mg)			
	TGL		GPAT	
	NI	I	NI	I
After 10 min. ischaemia	0.22±0.02	0.23±0.02	0.58±0.05	0.56±0.05
After isch. + 1 min reperfusion	_	_	0.56±0.05	0.43±0.05*
After isch. + 5 min. reperfusion	_	_	0.56±0.04	0.46±0.03*

Number of observation = 6

Values are means ± S.E.

Statistical significance v non-ischaemic value; *= $p<0.05$; **= $p<0.01$.

clear that the changes in enzyme activities brought about by ischaemia are mediated by β-adrenergic mechanisms.

Since the ischaemically-induced change in TGL activity is completely prevented by atenolol perfusion, changes in the activity of this enzyme were not studied during reperfusion.

During reperfusion, GPAT activity measured in the previously ischaemic area was significantly decreased compared to the activity measured after 10 minutes ischaemia. Thus, the reperfusion-induced fall in GPAT activity was not prevented by preperfusion with atenolol. Either an additional adrenergic drive occurs on reperfusion which is not blocked by atenolol (unlikely as no additional increase in TGL activity was seen on reperfusion in Table 3.13) or a component of the reperfusion-induced response which is not blocked by atenolol was responsible.

ii. Preperfusion with the Alpha$_2$ Adrenergic Antagonist Yohimbine.

Perfusion with the α_2 adrenergic antagonist yohimbine has been shown to increase noradrenaline release in response to sympathetic nerve stimulation (Langer, 1976). The effects of perfusion with the α_2 antagonist for 10 minutes before occlusion and thereafter throughout the experiment are shown in Table 3.20.

It can be seen that the non-ischaemic area showed a slightly elevated TGL activity after 10 minutes ischaemia and a slightly lowered GPAT activity after 1 and 5 minutes

TABLE 3.20.

The effect of continuous perfusion with the α_2 antagonist Yohimbine on the activities of Glycerol 3-phosphate acyltransferase (GPAT) and Triglyceride lipase (TGL) produced by 10 minutes ischaemia and subsequent reperfusion. Activities were measured in tissue from both the non-ischaemic (NI) and ischaemic (I) areas of the heart.

	ENZYME ACTIVITY (nmole/min/mg)			
	TGL		GPAT	
	NI	I	NI	I
After 10 min. ischaemia	0.24±0.04	0.30±0.03*	0.60±0.04	0.43±0.07*
After isch. + 1 min reperfusion	0.20±0.03	0.23±0.03	0.51±0.04	0.42±0.03*
After isch. + 5 mins. reperfusion	0.21±0.02	0.27±0.03*	0.55±0.07	0.42±0.0

Number of observations = 6

Values are means ± S.E.

Statistical significance v non-ischaemic value; *= $p<0.05$.

reperfusion compared to control values. These changes did not reach statistical significance but do suggest the possibility that a small increase in adrenergic activity occurs in the non-ischaemic area during occlusion and reperfusion.

In the ischaemic area, after 10 minutes ischaemia, TGL and GPAT activities were similar to the activities seen in the absence of the α_2 antagonist, ie. high TGL activity and low GPAT activity.

During reperfusion, however, the presence of yohimbine prevented TGL activity returning to normal in the previously ischaemic area. It appears that yohimbine has prolonged the ischaemic effect during reperfusion and kept TGL activity, in the previously ischaemic area, elevated.

GPAT activity remained low in the ischaemic area throughout the reperfusion period. This appears similar to the result seen in the absence of yohimbine. This could be due either to a continuation of the β-adrenergic drive (as TGL activity remained elevated) or to the reperfusion-induced fall in GPAT activity previously noted and which is not mediated by α adrenergic mechanisms.

iii. Pre-perfusion with the Alpha$_1$ Adrenergic Antagonist Doxazosin.

Doxazosin is a water-soluble derivative of the widely used α_1 adrenergic antagonist prasozin (Pfizer Central Research). Prasozin has been shown to be very effective in preventing the occurance of ventricular

arrhythmias due to coronary occlusion and subsequent reperfusion (Sheridan et al, 1980). The effect of continuous perfusion with doxazosin before, during and after ligation is shown in Table 3.21.

In the presence of doxazosin the change in TGL activity measured in tissue from both the non-ischaemic and ischaemic areas during occlusion and reperfusion shows a similar pattern to that seen in the absence of any adrenergic antagonist (Table 3.13). Similarly the fall in GPAT activity induced by ischaemia in the ischaemic area was similar in the presence or absence of doxazosin.

On reperfusion, as in the absence of doxazosin, GPAT activity in the previously ischaemic area was reduced compared to the activity measured in the non-ischaemic area. However, this value was not reduced when compared to the activity seen in control hearts and higher than the activity seen after 10 minutes ischaemia (Table 3.21). Indeed, after 5 minutes of reperfusion with doxazosin present, GPAT activity in the previously ischaemic area was comparable to that seen in the non-ischaemic area. This return to normal values was not seen with perfusion of Krebs/Hensleit buffer alone or in the presence of atenolol or yohimbine. Thus, it appears that the reperfusion-induced fall in GPAT activity was prevented by preperfusion with the α_1 adrenergic antagonist doxazosin. This provides strong evidence that the fall in GPAT activity seen on reperfusion of the ischaemic area is mediated by α_1 adrenergic mechanisms.

TABLE 3.21.

The effect of continuous perfusion with the α_1 antagonist Doxazosin on the activities of Glycerol 3-phosphate acyltransferase (GPAT) and Triglyceride lipase (TGL) produced by 10 minutes ischaemia and subsequent reperfusion. Activities are measured in tissue from both the non-ischaemic (NI) and ischaemic (I) areas of the heart.

	ENZYME ACTIVITY (nmole/min/mg)			
	TGL		GPAT	
	NI	I	NI	I
After 10 min. ischaemia	0.23±0.03	0.31±0.02*	0.66±0.04	0.48±0.04*
After isch. + 1 min reperfusion	0.21±0.02	0.28±0.01**	0.68±0.05	0.51±0.03
After isch. + 5 min. reperfusion	0.21±0.02	0.24±0.02	0.64±0.03	0.64±0.03

Number of observations = 6

Values are means ± S.E.

Statistical significance v non-ischaemic value; *= $p<0.05$; **= $P<0.01$.

DISCUSSION

Section 4.1.

The effect of adrenaline on the activities of triglyceride lipase, glycerol 3-phosphate acyltransferase and carnitine palmitoyl transferase.

The triglyceride content of the heart muscle appears to be in a dynamic state where the level of tissue triglyceride depends on the relative rates of esterification and lipolysis (Neely and Morgan, 1974). A considerable amount of evidence has accumulated to show the existence, in the heart, of lipases necessary for the hydrolysis of endogenous triglyceride stored at intracellular sites (Bjorntorp and Furman, 1962; Hamid et al, 1973 and Kriesberg, 1966).

The heart also contains a substantial activity of lipoprotein lipase which will, under appropriate physiological conditions, hydrolyze circulating triglyceride prior to entry into the cardiac cell (Robinson, 1970). The contribution of lipoprotein lipase to the activity measured as triglyceride lipase was reduced to a minimum by using a phospate buffer (pH 7.5) and avoiding any conditions known to stimulate lipoprotein lipase (such as the presence of serum cofactors) (Severson, 1979 and Al-Muhtaseb, 1982). Also, it might be expected that by administering heparin in the intraperitonial injection before the heart was excised, a large proportion of the extracellular lipoprotein lipase would have been removed (Borensztajn and Robinson, 1970a,b).

Numerous reports have described the hormonal control of intracellular triglyceride mobilization in heart tissue. Catecholamines have been shown to increase the rate of triglyceride mobilization in the perfused rat heart as demonstrated by increased rates of glycerol output (Williamson, 1964; Challoner and Steinberg, 1965; Kriesberg, 1966; Christian et al, 1969; Jesmok et al, 1977 and Severson et al, 1980) and decreased tissue levels of total triglyceride (Gartner and Vahouny, 1973; Crass et al, 1975 and Palmer et al, 1981).

The results reported in this study show that adrenaline perfusion can stimulate triglyceride lipase activity in the isolated rat heart. Bjorntorp and Furman (1962) have reported that a lipase was stimulated in the homogenate of rat hearts treated with adrenaline. Stimulation of TGL activity by adrenaline has also been observed in fat cell homogenates (Rizack, 1964; Huttunen et al, 1970; Corbin et al, 1970 and Steinberg, 1978).

In the same adrenaline-perfused hearts which showed a stimulated TGL activity, a concurrent fall in the activity of glycerol 3-phosphate acyltransferase was also seen. A similar inactivation of GPAT activity by adrenaline has been reported for fat cells (Sooranna and Saggerson, 1976,78), but not previously for the heart.

Adrenaline perfusion did not alter the activity of carnitine palimtoyl transferase in the perfused rat heart. No acute hormonal effects, arising from covalent modifications, have yet been shown on the activity of this enzyme in any tissue.

Section 4.2.

Mode of Adrenaline Action

Previous studies in the heart (Mayer, 1972; Kaumann and Birnbaumer, 1974 and Picken and Jarrett, 1975) have established that catecholamine-receptor interaction leads to increased adenyl cyclase activity and the generation of higher levels of cAMP. The mechanism by which cAMP has been shown to carry out its function as a second messenger in the transmission of hormonal signals is through the regulation of protein kinases found in various tissues (for review see Mayer, 1974).

It has been shown here that when homogenates or supernatants from perfused heart tissue were incubated in the presence of cAMP, ATP and cAMP-dependent protein kinase, the activity of TGL increased while the activity of GPAT decreased. The results suggest that TGL can be activated whilst GPAT can be inactivated by a phosphorylation reaction catalysed by cAMP-dependent protein kinase.

Activation of protein kinase by cAMP is thought to occur according to the following equation: (Brostorm et al, 1970)

$$2RC \text{ (inactive)} + 4cAMP \longrightarrow 2R\text{-}cAMP_2 + 2C \text{ (active)}$$

The enzyme exists as a tetramer with two regulatory (R)

and two catalytic (C) subunits. Binding of cAMP to the regulatory subunit of the inactive protein kinase (RC) causes a dissociation of the enzyme into the regulatory (R-cAMP complex) and an active catalytic (C) subunit. In the heart the protein kinase activity ratio (active enzyme/total enzyme), has been shown to correlate very well with the tissue cAMP levels under a variety of conditions (Corbin et al, 1970; Soderling et al, 1973 and Keely and Corbin, 1975). In work reported here, it has been shown that incubation of heart homogentate with the catalytic subunit (prepared from the holoenzyme by affinity chromotography and eluting with cAMP) results in activation of TGL and inactivation of GPAT by a time-dependent mechanism.

In adipose tissue it is well established that TGL can be activated by cAMP-dependent phosphorylation (Corbin and Krebs, 1969) and that this leads to an activation of lipolysis (Huttunen et al, 1970; Corbin et al, 1970; Khoo et al, 1976 and Belfrage et al, 1977). In rat heart however, Severson (1979) failed to detect any change in TGL activity in the presence of cAMP-dependent protein kinase. This was possibly due to his use of quick frozen tissue. In our hands, TGL activity measured in supernatants from frozen tissue was no longer activated by cAMP-dependent protein kinase.

With respect to GPAT activity there has been little previous evidence of adrenergic control. Only in rat adipose tissue has there been any reports of hormonal control. Nimmo and Houston (1978) reported that GPAT activity can be inactivated by cAMP-dependent protein kinase. However, Rider and Saggerson (1983a,b) failed to confirm this.

This present study shows that in perfused rat heart tissue GPAT activity can be inactivated by cAMP-dependent phosphorylation. Also in agreement with the results reported by Nimmo and Houston (1978) in adipose tissue, it was found that the microsomal but not the mitochondrial form of GPAT was inhibited by cAMP-dependent protein kinase phosphorylation. The fall in mitochondrial GPAT activity seen with adrenaline perfusion could not be explained by direct cAMP-dependent phosphorylation mechanisms.

If the activation of TGL and the inactivation of GPAT by cAMP-dependent protein kinase reflects the physiological process of adrenaline action, then TGL and GPAT in homogenates prepared from tissue previously perfused with adrenaline should already be largely in the activated (TGL) or inactivated (GPAT) form. This would mean that the activities should undergo little or no change when incubated with cAMP-dependent protein kinase. The results presented in Table 3.18 show this to be the case.

Attempts were made to dephosphorylate the enzymes from adrenaline-treated hearts by incubation under dephosphorylation conditions. Thus, homogenates or supernatants from hearts perfused with adrenaline were incubated with high Ca^{2+} and Mg^{2+} ion concentrations. Such conditions are known to favour phosphoprotein phosphatase activity (Severson et al, 1977). Under these conditions adrenaline-activated TGL activity was deactivated while adrenaline-deactivated GPAT activity was reactivated, ie. the effects of adrenaline were reversed. These results suggest the presence of a Ca^{2+} and/or Mg^{2+} dependent phosphoprotein phosphatase in heart tissue capable of

reversing the effects of adrenaline stimulation.

Thus, it is attractive to consider that adrenaline stimulates lipolysis through activation of TGL activity and decreases esterification through inactivation of GPAT activity. Both processes would be regulated by cAMP-dependent protein kinase and an integrated regulation of triglyceride mobilization and synthesis could occur.

Section 4.3.

The Role of Different Types of Adrenergic Receptor in the Regulation of Triglyceride Mobilization.

Naturally occuring catecholamines, namely noradrenaline and adrenaline, can interact with 4 different receptors on the cardiac cell surface; α_1, α_2, β_1, and β_2 adrenergic receptors (Lands et al, 1967; Berthelson and Pettinger, 1977 and Wikberg, 1979).

i. Beta Adrenergic Receptor

Beta agonists, such as isoprenaline, increase intracellular cAMP levels by activating membrane-bound adenyl cyclase, resulting in increased lipolysis after phosphorylation of triglyceride lipase in adipose tissue (Burns et al, 1971 and Fain, 1980). The increased mobilization of triglyceride in heart tissue as a result of catecholamine simulation is mediated via β-adrenergic

receptors (Shipp et al, 1973; Mayer, 1974; Hron et al, 1977 and Jesmok et al, 1977).

Perfusion of isoprenaline in our work resulted in a greater stimulation of TGL activity than was seen in the presence of adrenaline. Isoprenaline has been found to be more potent than adrenaline in increasing both intracellular cAMP levels and lipolysis in adipose tissue (Fain, 1973).

Similarly, perfusion of isoprenaline produced a greater fall in GPAT activity than was seen with adrenaline.

Thus, it appears that the changes in TGL and GPAT activities brought about by adrenaline perfusion are likely to result from the stimulation of β-adrenergic receptors. However, isoprenaline was more potent than adrenaline in producing an activation of TGL and an inactivation of GPAT.

ii. Alpha Adrenergic Receptor

Adrenaline and noradrenaline can also activate both α_1 and α_2 adrenergic receptors in the heart (Koblinger and Pichler, 1980,82; Drew, 1979 and Pichler and Koblinger, 1978).

Perfusion of the non-selective α agonist phenylephrine resulted in a fall in TGL activity and a rise in GPAT activity. This is consistant with the fall in cAMP levels measured by Keely et al (1977) when phenylephrine was perfused through the isolated rat heart.

In human and hamster adipose tissue a reduction in cAMP levels and a consequent reduction in the rate of lipolysis is mediated by α_2 adrenergic receptors

(Arktories et al, 1979,81 and Burns et al, 1981). Perfusion of the α_2 agonist clonidine in this work caused a fall in TGL activity and a rise in GPAT activity. This, again, is consistant with stimulation of the α_2 receptor causing a fall in cAMP levels. This would be produced by a decreased activity of adenyl cyclase (Jakobs, 1979; Jakobs and Schultz, 1980 and Jakobs et al, 1980,81) and would result in a reduced cAMP-dependent protein kinase activity.

A rather high concentration of clonidine was needed to achieve the maximum change in activities. This suggests that α_2 receptors are present at a low concentration and may not have a physiological role in the normal rat heart. However, their role might become more important under abnormal conditions such as in ischaemia where a fall in pH is known to favour the stimulation of α_2 receptors (McGrath, 1982).

Apart from post-synaptic α_2 receptors, which directly decrease adenyl cyclase activity, the rat heart also contains pre-synaptic α_2 receptors. Stimulation of these reduces noradrenaline release from sympathetic nerve terminals (Langer, 1974; Starke et al, 1975 and Dart et al, 1984a). Thus, perfusion of clonidine could also reduce intracellular cAMP levels indirectly by reducing the release of noradrenaline from the sympathetic nerve endings in the heart.

In the rat heart α_1 adrenergic receptors are found on both pre- and post- synaptic membranes (Koblinger and Pichler, 1980,82). The physiological significance of pre-synaptic α_1 receptors remains unclear but recent

evidence suggests that stimulation of this receptor can also reduce noradrenaline release (Ledda and Mantelli, 1984). Post-synaptic receptors have been shown to cause an increase in inotropy (Wagner and Brodde, 1978) possibly mediated by an increase in intracellular free Ca^{2+} concentration in the cardiac cell (Miura et al, 1978). This appears to be the result of an influx of Ca^{2+} from extra-cellular and/or intra-cellular storage sites (Berridge, 1975,80). It has been proposed (Michell and Kirk, 1981) that stimulation of phosphatidylinositol degradation is a primary and causal change involved in Ca^{2+} mobilization by α_1 agonists.

Perfusion of the isolated rat heart with the α_1 agonist methoxamine has been reported to increase glucose uptake, the phosphofructokinase activity ratio and lactate release (Clark and Pattern, 1981 and Clark et al, 1982) suggesting enhanced glycolysis. The effect of α_1 adrenergic stimulation on triglyceride mobilization has not been reported before.

The present work has shown that perfusion of the isolated rat heart with the α_1 agonist methoxamine had no effect on TGL activity but produced a persistant fall in GPAT activity. This affect has not been reported before and suggests that GPAT is sensitive to Ca^{2+} or some other α_1 mediated signal. In support of this hypothesis, Soler-Argilaga et al (1978) have reported that phosphatidate synthesis in the liver is reduced by an infux of Ca^{2+} ions.

iii. Mitochondrial and Microsomal GPAT Activity.

The changes in GPAT activity produced by perfusion with the β agonist isoprenaline and the α agonists phenylephrine, clonidine and methoxamine are localised in the microsomal (NEM-sensitive) fraction. The mitochondrial (or NEM-insensitive) fraction appears relatively unresponsive to these adrenergic agonists. This is consistant with the generally held belief that the endoplasmic reticulum is the prinicpal site of glycerolipid synthesis (Bell and Coleman, 1980).

iv. Carnitine Palmitoyl Transferase Activity.

Perfusion of the β agonist isoprenaline and the α agonist phenylephrine has no effect on CPT activity measured in the outer surface of the inner mitochondrial membrane. This agrees with the lack of any change in CPT_I activity produced by adrenaline perfusion and further supports the hypothesis that the activity of this enzyme is not regulated by acute hormonal effects in the rat heart.

Section 4.4.

Perfusion of Adrenergic Antagonists.

i. Beta Adrenergic Antagonist

Continuous perfusion of the cardioselective β_1 adrenergic antagonist atenolol results in a marked reduction in TGL activity (almost half that found in hearts perfused in the absence of atenolol). This suggests that control hearts were exhibiting some degree of adrenergic stimulation, even after 15 minutes perfusion. This could arise by local release of endogenous noradrenaline from sympathetic nerve endings during the perfusion. Overall the activity of TGL was seen to increase almost 4 fold from a level of 0.13 ± 0.01 under β-adrenergic antagonism to 0.42 ± 0.07 with β-adrenergic stimulation.

However, the fall in GPAT activity measured with continuous perfusion of atenolol cannot be explained by this method as a reduced adrenergic drive would be expected to increase GPAT activity. Thus, the fall in GPAT activity caused by perfusion with atenolol suggests that the reduction in activity caused by adrenaline may not be solely due to cAMP-dependent protein kinase effects mediated by the β-adrenergic system. It is possible that the α_1-adrenergic system is involved.

ii. Alpha Adrenergic Antagonism

Continuous perfusion of the α_1-adrenergic antagonist doxazosin and the α_2 antagonist Yohimbine did not result in any change in the activities of TGL or GPAT. This suggest that in the normal heart no appreciable α_1 or α_2 adrenergic drive exists.

Section 4.5.

The Effect of Ischaemia on the Activities of TGL, GPAT and CPT.

In the rat, ligation of the left descending coronary artery leads to ischaemia and infarction in a large area of the left ventricluar wall (Fishbein et al, 1980).

During acute ischaemia, increased release of noradrenaline from the sympathetic nerve terminals occurs (Shahab et al, 1972; Abrahamsson et al, 1983 and Schomig et al, 1984). This could be due to increased extracellular potassium (Hirche et al, 1980), acidosis (Eular and Lishajoko, 1961), hypoxia (Shahab and Wollenberger, 1967) and/or energy defficiency within the neurone itself (Wakade and Furchgott, 1968). Local release of noradrenaline from sympathetic nerve terminals is probably more important than

nerve-stimulated noradrenaline release during ischaemia, particularly during the early stages (Dart et al, 1984b and Schomig et al, 1984).

Exogenous catecholamines have been shown to stimulate myocardial lipolysis (Christian et al, 1969; Crass et al, 1975; Gartner and Vahouny, 1973; Hron et al, 1977 and Jesmok et al, 1977). It has been reported in the present work that both adrenaline and isoprenaline can increase lipolysis and decrease esterification by changes in the activities of the initial rate-limiting enzymes TGL and GPAT. During acute ischaemia and hypoxia increased lipolysis occurs (Opie, 1976 and Karwatowska-Kryna and Beresewicz, 1983) probably as a direct effect of increased noradrenaline release.

Catecholamine-stimulated lipolysis in the normally-perfused rat heart is probably mediated by increased cAMP levels via β-adrenergic stimulation (Christian et al, 1969 and Crass et al, 1975). In acute ischaemia, cAMP levels are known to rise markedly (Wollenberger et al, 1969 and Dobson and Mayer, 1973). After 2 minutes of ischaemia an increase in cAMP levels was seen in both the non-ischaemic and ischaemic areas of the rat heart (Krause and Wollenberger, 1967 and Krause et al, 1978). However, after 20 minutes, cAMP levels remained elevated in the ischaemic area but had returned to normal in the non-ischaemic area (Krause and Wollenberger, 1967 and Krause et al, 1978). By contrast, Podzuwiet et al (1978) found that cAMP levels were elevated only in the ischaemic area. This difference could be explained if the rise in cAMP levels in the non-ischaemic area is transitory.

Concurrent with the increased cAMP levels was an increase in the cAMP-dependent protein kinase activity ratio and the activities of phosphorylase kinase and phosphorylase (Krause and Wollenberger, 1967 and Dobson and Mayer, 1973). Pre-perfusion with the β adrenergic antagonist propranolol completely prevented the rise in cAMP levels and protein kinase activity ratio but only partly prevented the rise in phosphorylase kinase and phosphorylase activity (Krause and Wollenberger, 1967).

i. The Effect of Ischaemia on TGL, GPAT and CPT Activities.

The results reported here are in accord with the theory of an ischaemia-induced rise in cAMP levels and protein kinase activity. After 10 minutes occlusion TGL activity, measured in the ischaemic area, was increased by 50% compared to activity measured in normally-perfused hearts. A fall in GPAT activity (30%) occured in the ischaemic area. No change was seen in TGL or GPAT activity in the non-ischaemic area.

No change was seen in the activities of CPT_I or CPT_{II} or the total CPT activity in the ischaemic area after 10 minutes occlusion. Thus, both acute adrenergic challenge and acute ischaemic challenge do not appear to alter the activity of CPT.

Injection of 6 OH-dopamine at least one day previously has been shown to markedly reduce both the level of endogenous noradrenaline and the incidence of arrhythmias

during ischaemia and reperfusion in the cat (Sheridan et al, 1980) and guinea pig (Culling et al, 1984) heart. In the work reported here depletion of noradrenaline by injection of 6 OH-dopamine 24 hours before the hearts were excised prevented the rise in TGL activity and the fall in GPAT activity in the ischaemic area. This suggests that the changes in TGL and GPAT activities measured after 10 minutes ischaemia are caused by release of endogenous noradrenaline during ischaemia.

ii. Preperfusion with Adrenergic Antagonists

Preperfusion with the β antagonist atenolol, but not the α_1 antagonist doxazosin or the α_2 antagonist yohimbine, also prevented the ischaemia-induced changes in TGL and GPAT activities. Thus, it appears that these changes are mediated by the β-adrenergic receptor.

iii. Ischaemia-induced rise in Free Fatty Acids.

The accumulation of fatty acids and their acyl CoA derivatives in the ischaemic myocardium has been well documented (for review see Victor et al, 1984). Severson and Hurley (1982) have found that rat heart triglyceride lipase is inhibited by high concentrations of free fatty acids and fatty acyl CoA. These observations are in accord with previous work in our laboratory (Al-Muhtaseb, 1982). Thus, it may be expected that high levels of free fatty acids, produced during ischaemia, could inhibit TGL activity, overriding the activation produced by phosphorylation. This

would explain the results of Crass and Pieper (1975) who were unable to find any increase in triglyceride mobilization in the hypoxic heart.

Section 4.6.

Reperfusion of the Ischaemic Area.

It was Jennings et al (1960) who first noted that reperfusion of the ischaemic myocardium may not nescessarily be beneficial. They reported electrophysiological and structural damage associated with reperfusion. Since this observation our knowledge of the mechanisms and consquences of ischaemia and reperfusion have expanded enormously (for review see Hearse, 1977; Manning and Hearse, 1984 and Corr and Witkowski, 1984). However, whilst it is obvious that the ischaemic myocardium cannot recover without the restoration of adequate coronary flow, there is now a convincing body of evidence to suggest that reperfusion can induce a paradoxical extension of ischaemic damage.

There is considerable evidence of an increase in α-adrenergic activity during reperfusion of a previously ischaemic area (Sheridan et al, 1980). Using an anaesthetised cat model of coronary occlusion and reperfusion, Sheridan et al (1980) were able to show that whilst both β-adrenergic antagonism with propranolol and α-adrenergic antagonism with phentolamine reduced the incidence of ventricular arrhythmias during occlusion, only phentolamine was effective in reducing reperfusion-induced arrhythmias. Similar results were found with the specific

α_1 antagonist prasozin (Sheridan et al, 1980), thus indicating α_1 specificity to the antiarrhythmic effect of phentolamine.

i. The Effect of Reperfusion on the Activities of TGL and GPAT.

In this study, ischaemically-activated TGL activity returned to control values during reperfusion of the ischaemic area. However, GPAT activity measured during reperfusion was significantly lower than the activity measured after 10 minutes of ischaemia.

This reperfusion-induced fall in GPAT activity did not occur in hearts from rats preinjected with 6 OH-dopamine 24 hours previously. Thus, this change in GPAT activity may have occured as a result of endogenous noradrenaline release.

ii. Preperfusion with Adrenergic Antagonists.

Unlike the ischaemically-induced fall in GPAT activity, the reperfusion-induced fall was not prevented by preperfusion with the β antagonist atenolol. However, preperfusion with the α_1 antagonist doxazosin did prevent it. So did yohimbine

It has been shown earlier that perfusion of control hearts with the α_1-adrenergic agonist methoxamine resulted in a fall in GPAT activity but did not alter TGL activity. Thus, there is good evidence to suggest that, on reperfusion, an increased α_1 adrenergic stimulus results in a fall in the activity of GPAT and, therefore, reduces

the capacity of the tissue to re-esterify endogenous fatty acids. This can be prevented by removal of endogenous noradrenaline (by 6 OH-dopamine injection) or by α_1 antagonism (with doxazosin).

Recent work has suggested that α_1 adrenergic antagonism may not be a specfic aspect of the benefits of prasozin in reducing the occurance of ventricular arrhythmias (Thandroyen et al, 1983 and Dukes and Vaughan-Williams, 1984). They suggest that these agents may have a local membrane-stablising effect. However, the fact that hearts from 6 OH-dopamine treated animals did not show a reperfusion-induced fall in GPAT activity does suggest that this change is mediated by adrenergic receptor mechanisms and that the evidence is in favour of doxazosin acting via its α_1 adrenergic antagonist properties.

It is known that α_1 adrenergic agonists increase cytosolic Ca^{2+} in the heart (Muira et al, 1978). Recently, it has been shown that reperfusion of the ischaemic myocardium is accompanied by an increased Ca^{2+} influx mediated by α_1 adrenergic mechanism(s) (Sharma et al, 1983). An increase in Ca^{2+} concentration has also been implicated in the reduction of phosphatidate synthesis by glucagon in hepatocytes (Soler-Argilaga et al, 1978). Therefore, it would appear possible that a reperfusion-induced rise in Ca^{2+} influx into the cardiac cytoplasm, mediated by α_1 adrenergic mechanism(s), causes a fall in GPAT activity and hence in esterification.

iii. Preperfusion with an Alpha$_2$ Adrenergic Antagonist.

Presynaptic α_2 adrenergic receptors are powerful modulators of noradrenaline release (Langer, 1976). Yamaguchi et al (1977) have shown increased noradrenaline overflow from the heart with α adrenergic antagonism. The α_2 antagonist, yohimbine (Tanaka and Starke, 1980), has been shown to potentiate noradrenaline release from the ischaemic area of an anaesthetised dog when intermittant left ganglion stimulation was applied (Forfar et al, 1983).

In our isolated system, the presence of yohimbine did not increase the changes in TGL or GPAT activities seen after 10 minutes ischaemia. However, on reperfusion, the α_2 antagonist did appear to postpone the return of TGL activity to control values, possibly by inhibiting neuronal reuptake (Forfar et al, 1983).

GPAT activity remained low during reperfusion with yohimbine present and did not recover to control values. This could be due to either a prolonged β adrenergic drive or the reperfusion-induced fall in GPAT activity seen in the absence of yohimbine.

Although no statistically significant changes in enzyme activities were seen in the non-ischaemic area, there was some evidence of a rise in TGL activity and a fall in GPAT activity in this area when yohimbine was present. Also, perfusion of the β antagonist atenolol, which has been shown to reduce TGL activity in control hearts did not reduce the

activity in the non-ischaemic area. These results would be consistant with a small, transient, rise in cAMP levels in the non-ischaemic area after coronary artery ligation, as shown by Krause et al (1978).

Section 4.7

Conclusion

The work reported in this thesis has shown how catecholamine and ischaemic challenge can effect triglyceride mobilization in the heart via the activities of triglyceride lipase and glycerol 3-phosphate acyltransferase. Adrenaline has been shown to activate TGL activity and inactivate GPAT activity thus facilitating triglyceride mobilization. This effect is probably mediated by activition of the β adrenergic system, causing an increse in cAMP levels and an increase in the activity of cAMP-dependent protein kinase.

Stimulation of α_2 adrenergic receptors produces a fall in TGL activity and a rise in GPAT activity. This is probably mediated by a fall in cAMP levels. Stimulation of α_1 adrenergic receptors does not alter the activity of TGL but does produce a fall in GPAT activity.

After 10 minutes ischaemia TGL activity in the ischaemic area is higher than control values whilst GPAT activity is lower. This is probably caused by ischaemically-induced release of endogenous noradrenaline from sympathetic nerve terminals. These changes in TGL and GPAT activities can be prevented either by depletion of endogenous noradrenaline by preinjection with 6 OH-dopamne or by preperfusion with the β adrenergic antagonist atenolol.

During reperfusion of the ischaemic area, TGL

activity returns to control values. GPAT activity, though, is further reduced. This fall in GPAT activity can be prevented either by preinjection with 6 OH-dopamine or by preperfusion with the α_1 adrenergic antagonist doxazosin. Thus, the reperfusion-induced fall in GPAT activity appears to be mediated by an increased α_1 adrenergic drive during reperfusion.

Further Work

i. Investigation of the effects of changes in calcium concentration in the perfusate on TGL and GPAT activities during normal, ischaemic and reperfusion conditions.

ii. Investigation of the effects of anti-arrhythmic calcium entry blockers on the activities of TGL and GPAT after ischaemia and reperfusion.

iii. Investigation of the effect of the presence of fatty acids in the perfusate on the activities of TGL, GPAT and CPT and the ischaemia and reperfusion induced changes in enzyme activities.

iv. Investigation of the use of different concentrations of adrenaline and other alpha and beta agonists, possibly by the use of cardiac myocytes.

v. Investigation of the effects of hypoxia, compared to ischaemia, on the enzyme activities.

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